

Trust and the bioethics industry

Radical developments in biology often appear to represent new conflicts with moral principles. Such perceptions are frequently misconceived, but independent consideration of ethical implications is beneficial.

There has been something both reassuring and disturbing about the conviction with which the leaders of virtually every advanced industrialized nation have taken a strong public stance against the cloning of human beings, reflected most recently in last weekend's statement from the summit meeting of the 40 member states of the Council of Europe (see page 656). The reassuring part has been their desire to confirm a commitment to human values that could, at least in theory, be challenged by the unrestrained application of certain biomedical advances. The disturbing aspect is the extent to which an eagerness to grab the moral high ground risks short-circuiting two important debates: about the many positive aspects of the science involved, and about the most effective ways of integrating public concern into a viable science policy.

One interpretation of the anti-cloning warnings of individuals ranging from US President Bill Clinton to Federico Mayor, the director-general of Unesco, is that they reflect a growing global distrust of the religious, political and scientific institutions traditionally responsible for addressing moral and ethical issues. Further evidence for this comes from the rapid rise of the professional discipline of bioethics. Until recently restricted to debates on the activities of the medical profession, this has since established itself as a new and influential expression of public concern about the many ways in which modern genetics and related fields will impinge on our lives (see pages 658–663).

There is, of course, a danger in overreacting to public concerns, particularly when these may be little more than off-the-cuff responses to an unexpected development — such as the announcement of the cloning of Dolly the sheep. Excessively rigid legislation could stifle potentially important research with beneficial implications; such was the message of members of the Federation of American Societies of Experimental Biology, who recently volunteered to accept a moratorium on human cloning as such. And others, such as those opposed in principle to abortion or animal experiments, may seek to exploit the concern for their own dogmatic ends.

But there is also a danger of another kind, namely that politicians and others may be tempted to resort to traditional institutional remedies, such as expert advisory panels and tough-sounding legislation, without recognizing the extent to which these are no longer sufficient to secure public trust. One of the key lessons to emerge from recent debates about social risk is that trust cannot be assumed to be created by reassurance from on high or by quoting the conclusions of scientific 'experts'. Rather, it is created through a complex set of social interactions — with participants ranging from laboratory researchers to journalists — whose outcome no single authority is in a position to determine or control.

This shift has important implications for the way in which we measure, represent and seek to manage risks, from human cloning to AIDS. The traditional sciences still have a critical role to play in establishing some of the parameters in such debates; solutions based on a misinterpretation of experimental evidence are almost bound to fail. But the process of risk management, as a report published earlier this year by the US National Academy of Sciences points out, is not a linear one built on scientific arguments, but a complex, iterative process in which many actors and disciplines must be involved.

Where does bioethics fit in? It would be as wrong to look on bioethics as a source of answers to difficult social questions as it would be to pretend that such answers will emerge from a purely scientific analysis or technical risk assessment. But, as our survey highlights, bioethicists do have several important functions. One is to provide a vehicle for analysis away from the spotlight of immediate political pressure — claims by scientists that a particular development, representing significant new power to manipulate nature, in itself carries no new ethical implications require independent scrutiny. A second is to formulate questions and concerns as a result of this analysis in a language that can, in parallel with scientific arguments, be integrated into the policy-making process. In doing so, bioethics can, perhaps most importantly, help to forge a new, democratic discourse about science in which the voices of all have their appropriate place. □

Vaccines at risk

An imaginative attempt to tap Asian resources for the benefit of the developing world deserves more support.

Sadly, the developed world seems to be so unwilling to donate money to the prevention of disease in the developing world that backers of two worthy initiatives to develop vaccines for children may end up fighting over the same small pot of funds (see page 655). Promoters of the International Vaccine Institute in Seoul, who realized that there is new money in the (until recently) booming economies of Asia, have succeeded in winning substantial Korean government support for the institute's construction and 30 per cent of its running costs. But some officials of the World Health Organization (WHO) are concerned that, in pursuing the remaining \$10 million or so a year that will be needed to run it, the new institute may eat into the small \$30-million pie of the WHO-backed Children's Vaccine Initiative

which is itself responsible for the institute.

Both initiatives seek to provide vaccines to prevent disease in children with, in the case of the Seoul institute, a focus on the developing world, and in particular Asia. Is the world so poor it cannot afford the \$40 million required to support both initiatives? There were some understandable — but misguided — concerns that the institute was a Korean attempt to tap into Western vaccine technology for the benefit of Korean industry. But the establishment of a distinguished international board of trustees to oversee the institute and a clear statement that it will not engage in the sale of vaccines should dispel such fears. The developed world, including Japan and the United States, should chip in with support. □



French ministries in argument over release of asbestos report

[PARIS] Tension is growing between French government ministries over allegations that the publication of a report on the risks of asbestos prepared by an expert panel of INSERM, the national biomedical research agency, has been blocked by INSERM itself, on the instructions of the ministry of national education, research and technology.

Given the enormous economic, social and political stakes surrounding asbestos, the report is the most politically sensitive prepared by INSERM's service of 'collective expertise', created in 1994 to provide quick answers to contemporary questions of public health (see *Nature* **368**, 468; 1994).

A short summary of the 560-page report, *Effects on health of the principal forms of asbestos*, was hastily released in July 1996 just before the government announced a ban on all forms of asbestos. A working draft of the full report was made available for consultation, pending publication.

But the research agency now says that it has decided not to publish the final report as a book as originally agreed, but simply to place a notice in the next issue of its in-house magazine, *INSERM Actualités*, informing readers that copies of the final report can be bought directly from INSERM.

The report was commissioned from the INSERM panel by the department of labour relations (DRT) of the ministry of employment and solidarity, and the Direction Générale de la Santé of the ministry of health, on the basis that the final report would be published. The ministry of national education, research and technology, which shares responsibility for INSERM with the ministry of health, had no direct involvement.

Claude Allègre, the minister for national education, research and technology, has been an outspoken critic of the report.

In a magazine article written before he became science minister, Allègre argued that the risks of asbestos had been overestimated, and described the INSERM report as "not glittering, either by its scientific rigour, or by its courage". He has also contested the FFfr1.2 billion (US\$200 million) plan to decontaminate the huge Jussieu campus in central Paris, where his laboratory is located, arguing it is unnecessary and perhaps even dangerous.

Several independent sources allege that the office of Claude Griscelli, director general of INSERM, blocked publication of the report while awaiting authorization from the science ministry. The order not to publish the report "came directly from the ministry", claims one INSERM official, an account confirmed by officials at the DRT. Similarly,



Battleground: plans to remove asbestos from the Jussieu complex in Paris have previously been attacked by the current research minister as unnecessary and perhaps even dangerous.

instructions that INSERM should simply announce that unprinted versions of the report were available came from the science ministry, according to one INSERM official.

Allegations of political interference are denied by Suzy Mouchet, a spokeswoman for INSERM. She agrees that there may have been discussion of the report at the science ministry given Allègre's "particular sensitivity" to the issue, but argues that there is a "subtle distinction" between "discussing it at the ministry, and demanding the authorization of the ministry". She says: "It is normal that there are discussions between Griscelli and Allègre's cabinet."

One DRT official says, however, that the DRT is angry about INSERM's handling of the report, and that it intends to write to the

agency within the next few weeks to protest at its failure to publish the report as agreed, and to demand that the decision be revoked.

The official explanation for INSERM's decision is that it considers the market for the final report to be too small to justify the estimated cost of FFfr60,000–FFfr70,000 of printing, particularly as more than 2,000 copies of the summary have already been distributed.

Vincent Courtillot, Allègre's principal adviser and director of the Jussieu-based Institut de Physique du Globe, says that while he believes the report should be made public, "we don't think it needs to be published as a book [on the grounds of costs]".

Mouchet also argues that the availability of the report will be advertised more widely than it would have been had it been pub-

nature

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- **Sir Ron Oxburgh** (Rector of Imperial College, London, and a member of the Dearing Committee)
- **Mark Ferguson** (School of Biological Sciences, University of Manchester)
- **Jean-Patrick Connerade** (Department of Physics, Imperial College, London; member of the executive council, Save British Science)
- **Dame Bridget Ogilvie** (Director, The Wellcome Trust)
- **Brian Fender** (Chief Executive, Higher Education Funding Council for England)
- **David Triesman** (General Secretary, Association of University Teachers)

lished, as more than 12,000 copies of *INSERM Actualités* are distributed. She adds that whereas the summary of the report could be easily understood, the full report is highly specialized and could be misinterpreted by "people who don't have the competence to understand it." "There is a big risk in distributing a report of this nature to a wide population," she says. "It poses a problem in terms of communication."

But such arguments are viewed sceptically by the DRT. One official points out that publication of the report was agreed in its contract with INSERM, adding that the DRT "will not accept a unilateral decision taken by INSERM". Moreover, while INSERM has played down the difference between making the report available and publishing it, the DRT considers the distinction is important.

One official from the DRT points out that as it stands the report is "grey literature". This makes it more difficult for scientists elsewhere to obtain it, and risks downgrading its status. "The report must be confirmed by publication; not to do so would throw doubts on its validity," particularly as its contents have been challenged internationally.

Indeed, the French ban, and the contents of the full INSERM report, have already been vigorously contested by the Canadian federal government, and the government of Quebec in particular (see *Nature* **385**, 379; 1997). The French have been criticized for including chrysotile asbestos in the ban; the adequacy of extrapolations of toxicity to lower levels of exposure has also been challenged.

Failure to publish the report would "undermine" the series of measures taken by the government on asbestos, says the DRT official. These were partly justified on the basis of the report's contents. The official claims INSERM's handling of the report raises questions about its "independence" from its political masters. He said: "This is a very serious matter for us, it is shocking."

Researchers involved in the preparation of the report are also unhappy. "It is the first time in my life that I have seen INSERM ask the question 'should we publish or not a report that might not please the minister,'" says one. "It is of extraordinary gravity."

One member of the scientific board of INSERM says the handling of the publication of the report has been "unusual". "First we say that here we have independent scientific advice, and then we don't publish it because of political reasons," the researcher says, although acknowledging that the report is the most "politically explosive" INSERM has been asked to produce.

Observers point out that Griscelli, who is close to the neo-Gaullist RPR party and whose appointment was widely considered to be political, is in a vulnerable position as his post as director general has been in question since the Socialists came to power in the general election in June.

Declan Butler

US energy official departs with a 'get real' warning

[WASHINGTON] The senior official responsible for environment, health and safety at the US Department of Energy leaves Washington this week with a warning for scientists: engage with the communities you live in, or face oblivion.

Tara O'Toole, a physician who has spent four years crusading against entrenched practices as an assistant secretary at the department, says scientists need to wake up to what is happening outside laboratory gates.

O'Toole played a key role in initiating a huge, cross-government investigation into the human subjects research that took place in the United States during the Cold War. More recently, she upset some scientists at the Brookhaven National Laboratory with her aggressive approach to environment problems there.

O'Toole practised and taught medicine in Baltimore, Maryland, and then worked on environmental health issues for the congressional Office of Technology Assessment before joining the administration. She was one of a cadre of radical officials sprinkled by President Bill Clinton across his first administration. As this group tires and departs — four years is considered a long time in such positions — it is being replaced, in general, by a more orthodox class of Washington official. O'Toole's successor has yet to be named.

"We 'baby-boomers' just haven't been realistic about how difficult it is to change the world," O'Toole reflects. "I hope more people come forward to do this kind of work."

Scientists need to accept that "the political process isn't fair" and yet still engage with it, she argues. "It is very important that scientists get in the game. The community of scientists better do what needs to be done to assure the public that its programmes are properly run."

O'Toole led the Department of Energy's public response when a tritium leak was discovered at the Brookhaven National Laboratory on Long Island, New York, earlier this year (see *Nature* **386**, 3; 1997). The department responded with high-profile investigations into the management of the laboratory, and soon sacked its managing contractor, Associated Universities Incorporated.

Some scientists at the laboratory believe that the department overreacted, giving encouragement to critics who would like to see it shut down. Nick Samios, who retired as laboratory director in March, said at the time: "Tara is trying to be helpful, but it isn't wise to constantly hold these press conferences. He noted that every one of them led to negative publicity for the laboratory."

O'Toole counters that the department



O'Toole: scientists must "get in the game".

acted to save the laboratory from itself by piercing its complacency about the perceptions of people outside. She says she sympathizes with Brookhaven scientists "to some extent", especially with graduate students whose projects were wrecked by the suspension of reactor-based research there.

"But the root problem was that scientists at Brookhaven were not engaged with the political realities of the community in which they live," she says. "I don't think they yet grasp the peril to the laboratory that is posed by the tritium plume — it isn't a threat to public health, but it sure is to the laboratory."

She also thinks that lessons learned at Brookhaven will eventually come into play for biology, as it comes to resemble 'big science'. "The Human Genome Project is the beginning of 'big biology,'" O'Toole says. "Science is no longer a cottage industry." She predicts biologists engaged in big science will have to engage the public more effectively.

Energy department watchers say O'Toole will be sorely missed. "She was very thorough and committed and brought an incredible amount of technical knowledge to the job," says one Congressional staffer.

Asked who will continue where she left off at the department, O'Toole replies dutifully that Federico Peña, the energy secretary, and his deputy and probable successor, Elizabeth Moler, "care about openness" and will continue to push for it. But neither Peña, a career politician, nor Moler, a Washington lawyer, is likely to upset the apple-cart at DoE in the manner of O'Toole or Hazel O'Leary, the previous energy secretary.

O'Toole's specialized knowledge helped turn O'Leary's vision of a more open department into reality — especially through the investigation into human subjects research, which eventually embraced the entire federal government. "They fought with the culture [of the laboratories], and it outlasted them," says the staff member. "But they did make a difference."

Colin Macilwain

Transatlantic talks on space telescope

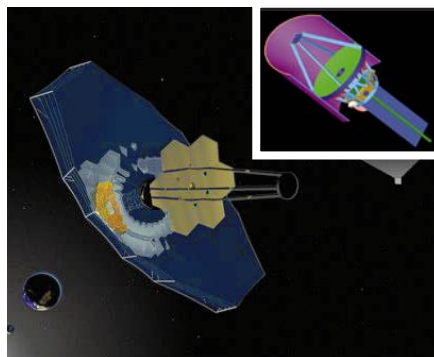
[WASHINGTON] Representatives of US and European space agencies will meet later this month to begin formal discussions on collaboration on a Next Generation Space Telescope (NGST), to be launched in the first decade of the next century.

The instrument would be optimized for infrared studies of very young galaxies, and would succeed the Hubble Space Telescope as the main observatory in space.

The US National Aeronautics and Space Administration (NASA) has been conducting preliminary feasibility studies for the NGST project since spring 1996 (see *Nature* 381, 465; 1996). The European Space Agency (ESA) has yet to begin a formal study, although ESA officials have told their US counterparts that Europe is interested in participating in the project, and might contribute as much as \$200 million. That level of investment would place it in the category of ESA's new 'flexible' missions.

NASA hopes to build the telescope for around \$500 million, beginning construction in 2003, with a launch planned for 2007. John Mather, the NGST project scientist at NASA's Goddard Space Flight Center in Maryland, says the agency will spend an additional \$150 million–\$200 million over the next five years developing technologies for the telescope, including lightweight deployable optics. Launch and operational costs would also be extras, says Mather.

NASA will spend about \$20 million in



Rival bids: two possible designs of new facility, from TRW and (inset) Lockheed-Martin.

1998 on NGST studies and technology development. Two industry contractors, Ball Aerospace and TRW, are already doing mission architecture studies in which the telescope would be in a stable orbit at least 1.5 million kilometres from Earth, where it would stay very cold. The mirror is likely to be deployable in segments that would have to be assembled in space.

NASA's designs are well ahead of ESA's, says Jean-Pierre Swings of the Institut d'Astrophysique at the University of Liège in Belgium, who heads a task force that will soon report to ESA on options for participating in the project. Swings says that European astronomers have a "very strong wish to be involved". His task force of six scientists from outside the agency will recommend that ESA immediately begin technical studies, with

the intention of eventually merging its design work with that of NASA. ESA's possible contributions include supplying an Ariane rocket launch, building ground stations and supplying science instruments.

According to Swings, the United States has a clear lead in infrared detector technology, but European scientists have other areas of expertise that could be useful. ESA's European Space Research and Technology Centre in the Netherlands is working on a superconducting-tunnel-junction camera that can simultaneously take images and spectra at ultraviolet, optical and near-infrared wavelengths. The instrument lost out in a recent competition for addition to the Hubble telescope in 2002, but could possibly be adapted for the new telescope.

The ESA–NASA meeting planned for 30 October in Washington is the beginning of a lengthy process that could eventually produce a memorandum of understanding about cooperation on the NGST.

Swings says it would not need to be a 50:50 partnership for European astronomers to benefit. Europeans have about 20 per cent of the viewing time on the Hubble telescope, which seems to serve the space community well, he says.

NASA hopes to fund its part of the NGST project by scaling down operations of the Hubble telescope. By not servicing Hubble after 2002, the agency could save two-thirds of its operating budget. **Tony Reichhardt**

Political crisis threatens delay to revitalized Italian programme

[MUNICH] The fall of Romano Prodi's Italian government last week could delay the adoption of the Italian Space Agency's proposed five-year plan, which is set almost to double the amount of money spent on basic research.

The details of the IL6.5 trillion (US\$3.8 billion) space plan for 1998 to 2002, which was approved by the government last month and which will make Italy the third highest spender on space science in Europe, are due to be formally submitted to parliament at the end of this month.

But if President Oscar Luigi Scalfaro chooses to call a general election rather than form an interim government, parliamentary approval of the 1998 budget, of which the plan is a part, is unlikely before the end of the year. That would delay new initiatives for the first part of next year.

And the plan includes many new and important initiatives. The space agency, ASI, has regained most of the credibility it lost in the early 1990s when it was dragged down by financial crisis, political scandal and general mismanagement, and when

payment of approved research grants ceased because of a bizarre and protracted battle about the proportion of the ASI budget that should legally be dedicated to basic research (see *Nature* 357, 351; 1992).

The new plan allocates more than 20 per cent of the budget to science, nearly doubling the science budget allocated by ASI previously. Money will be divided equally between national activities and activities associated with the European Space Agency (ESA). National activities comprise four programmes: small scientific satellites; planetary exploration; scientific uses of the Space Station; and investigator-initiated basic research.

For the first time the scientific community has been directly involved in defining the ASI science plan, says Giovanni Bignami, the agency's science director. The community will also be directly involved in the selection of missions. A call for ideas for small satellite missions was put out in May. Around 60 proposals have been received by ASI. About ten of these will be chosen by ASI for presentation at an open meeting in early

December, where the scientific community will select five for parallel feasibility studies to be completed next year. The eventual winner may expect a launch around 2002.

The planetary exploration programme will fund participation of Italian scientists in missions initiated by ESA and the US National Aeronautics and Space Administration (NASA). For example, ASI is discussing with NASA the possibility that Italian scientists be given access to surface-science robotics experiments in NASA's Mars programme, in view of NASA's recent invitation to Italy to contribute to the Mars programme's telecommunications system.

ASI has also doubled its budget for scientific exploitation of the International Space Station. "The station is here to stay," says Bignami. "So we must make the best possible use of whatever opportunities it offers for science."

A further fifth of the space plan's budget will be devoted to Earth observation, and an eighth to the development of a national launcher, able to put payloads of up to one tonne into a low-Earth orbit. **Alison Abbott**

Mentally disabled research subjects 'need protection'

[WASHINGTON] When the US National Bioethics Advisory Commission (NBAC) convenes again on Sunday (19 October), it will wrestle with a difficult matter on which it is due to report by January. The commission's human subjects subcommittee, which meets the same day, is charged with working out how to provide adequate protection for mentally disabled research subjects, for whom no specific protection exists under federal regulations.

Two national commissions have recommended that the government adopts such protection — which already exists for pregnant women, fetuses, prisoners and children. But an early effort to introduce it was abandoned after an outcry from researchers. Patients' advocates say this has led to widespread abuses, and are calling for stringent government measures to be adopted.

"Highly speculative, relapse-producing experiments may have served the interests of investigators, but they undermined the best medical interests of the subjects," says Vera Hassner Sharav, director of Citizens for Responsible Care in Psychiatry and Research.

The seriousness of the advocates' cause was brought home to members of NBAC's human subjects subcommittee at a meeting last month where they heard harrowing testimony from former research subjects. They alleged cursory, inadequate or nonexistent informed-consent procedures, abrupt withdrawal of medication, inducing psychosis, and restraint in locked wards.

Although several cases presented at the meeting involved private facilities, others involved research financed by the National Institute of Mental Health (NIMH), including one study at the institute itself in Bethesda, Maryland. There, a former subject said, he was presented with a notebook of consent forms three to four inches thick. "I was given no opportunity to read them [or] consider them. The doc turned the pages and I signed."

Such a process "could not in a million years be characterized as informed voluntary consent," said Alexander Capron, an NBAC member who is a bioethicist at the University of Southern California.

Rex Cowdry, acting deputy director of NIMH, said the institute took the allegations "seriously". He said the institute "is already actively involved in a broad examination of how we can assure that our research participants are well-informed".

But some NBAC members said it was important that protection for subjects should not cripple research. "It also can be unethical not to do research," said Arturo Brito, a paediatrician at the University of Miami. **M.W.**

Row over alternative medicine's status at NIH

[WASHINGTON] Six years after it was set up on the recommendation of a congressional committee, the Office of Alternative Medicine (OAM) at the US National Institutes of Health (NIH) is again under close public scrutiny, this time over the proposal that it should be elevated to a grant-giving centre.

Senator Tom Harkin (Democrat, Iowa), who as head of a key subcommittee responsible for funding the NIH was instrumental in OAM's creation in 1991, is now suggesting that the \$12.5 million office be upgraded to a National Center for Complementary and Alternative Medicine Research.

But the proposal has run into stiff opposition from parts of the scientific establishment who, deeply sceptical of many of alternative medicine's claims, want OAM abolished. At a hearing last week of the Public Health Subcommittee of the Senate Labor and Human Resources Committee, the critics' views received close attention.

Harkin believes that NIH bias against the OAM has rendered it nearly ineffectual. OAM was created to investigate and validate alternative therapies, but hasn't "investigated and validated one darn thing" and needs the institutional power to do so, he said.

OAM grants must at present be funded in collaboration with other institutes. According to Harkin, this means that peer-reviewers with no interest in alternative medicine give proposals short shrift.

He pointed as evidence to budget figures from 1996, when an estimated \$43.7 million of NIH's \$11.9 billion budget was spent on alternative medicine research. Harkin charged that NIH's director, Harold Varmus, has requested that OAM's own budget for this year be cut by 40 per cent, to \$7.5 million.

"What signal does that send about the interest of the head of NIH" in alternative research, Harkin demanded? He says the solution is to give OAM the power of an NIH centre, with authority to recruit its own peer-reviewers and award its own grants.

But Bill Frist (Republican, Tennessee), a cardiac surgeon and the subcommittee chairman, said scientists fear "you are going to get unsafe, ineffective products, therapies, procedures" if people who believe in alternative medicine are made peer-reviewers.

OAM's critics argue that alternative therapies should be examined by peer-reviewers within NIH's existing institutes. Frist has received a letter from Nobel laureates, including Paul Berg of Stanford University and Allan Bromley of Yale, urging that OAM's effectiveness be rigorously scrutinized before an upgrade is even considered.

"To elevate the Office of Alternative Med-

My, that looks painful—have you tried acupuncture?



icine to the status of a national centre without first examining its strengths and weaknesses would risk amplifying existing problems," the laureates wrote. Bromley, science adviser to former president George Bush, has elsewhere accused OAM of lending credibility to practices that "more clearly resemble witchcraft than medicine".

One witness at the hearing, Robert Rich, vice-president at Baylor College of Medicine in Houston, Texas, testified on behalf of the Association of American Medical Colleges that upgrading OAM to a centre would "at least double" its administrative costs. "Use the experience and talent of the existing institutes" to evaluate research on alternative therapies, he said.

But another witness, James Gordon, the director of the Center for Mind-Body Medicine at Georgetown University in Washington DC, and the first chairman of the advisory council at OAM, said the public would be far better served by an expanded OAM.

Gordon called the office's current budget and staff size "completely inadequate", and urged a budget of \$100 million–\$150 million. He said that, among 125 reviewers on NIH's 26 standing review committees, not one had a degree or licence in alternative medicine fields such as chiropractic and acupuncture.

Whatever the scientists' views, it is clear that alternative therapies are intensely interesting to the US public. One-third of respondents in a 1993 study in the *New England Journal of Medicine* said they had used an alternative therapy in the previous year. And OAM receives about 1,200 calls from the public each month, a figure at least equal to those to institutes with 100 times its budget.

OAM's profile is likely to remain high. Next month it will host a consensus conference on acupuncture on the NIH campus in Bethesda, Maryland. **Meredith Wadman**

'No need for haste' on Japan's fast reactor

[TOKYO] Japan should continue research and development work at its experimental fast-breeder reactor Monju, but should postpone decisions on the construction of a demonstration reactor. These are the main conclusions of a preliminary report by an advisory body of the Atomic Energy Commission.

The report by the Special Committee on Fast-Breeder Reactors says energy prospects for Japan for the next century are problematic, and that keeping options open for future generations is "imperative". But it says it is too early to decide on a demonstration fast-breeder reactor.

Admitting that some committee members disagree with its conclusion, the report argues there is evidence that the main problems of fast-breeder reactors — including safety and economic feasibility — may be solved eventually. It concludes that "it is too early for a decision" on the demonstration reactor, and that "there is no need to abandon fast-breeder development at the present stage".

On the prospects for commercial fast-breeder reactors, the report says that "a decision on the construction of a prototype reactor should reflect experiences gained in operating the Monju reactor". But that decision should depend as much on economic feasibility and the evolution of energy markets as on technical criteria.

It was evident well before a recent meeting of the special committee that enthusiasm in Japan for fast-breeder technology had almost evaporated. The report's recommendations "do not express our preferred choice", said committee chairman Junichi Nishizawa, former president of Tohoku University in Sendai. "But there is simply no alternative."

If the report is accepted, Japan would follow the United States and Europe in beating a retreat from a commitment to build fast reactors. Doubts first emerged in the United States in the early 1980s, fuelled by concern over the use of plutonium, as well as evidence that the price of uranium appeared unlikely to rise to a point where fast reactors would be economically competitive.

Last year, France shut down its prototype reactor Superphénix, and announced that it would in future only be retained as a research reactor. Earlier this year, however, the new socialist government announced it would be closed for good (see *Nature* 387, 646; 1997). A project to build a European Fast Reactor was also abandoned when the United Kingdom and Germany withdrew in 1992.

Japan's special committee was set up in response to increasing public discontent with Japanese nuclear policy after an accident at Monju in December 1995 (*Nature* 378, 654; 1995). It is one of the first govern-

ment advisory bodies to include well-known critics of Japan's nuclear fuel cycle policy (*Nature* 386, 209; 1997).

Hitoshi Yoshioka, a historian who trained as a physicist and who represents the critics, says he is satisfied some of his propositions have been incorporated in the report. He cites his request for a broad external evaluation of experiences and data gained by operating the Monju reactor as one example.

But Yoshioka adds that, while democratization of decision making at the Atomic Energy Commission is in progress, there is still a long way to go. He finds it especially embarrassing that the future of the Power Reactor and Nuclear Fuel Corporation, also known as Donen, which operates Monju, was decided before the committee on fast-breeder reactors could reach its conclusions.

In late July, an advisory panel set up by the Science and Technology Agency (STA) proposed a reorganization to create a new body to take over most of Donen's core activities, but would close — or spin off — less central ones, including uranium enrichment and overseas logging and mining operations.

Focusing mainly on organizational and management issues, the panel's report delib-

erately omitted questions of future research and development strategies or nuclear fuel cycle policy in general.

With some 3,200 staff — including a substantial number of engineers from the private sector — Donen is by far the largest research complex under the STA. The demise of the fast-breeder option would not only have raised additional questions about the future of Donen but would also have undermined STA's standing, already under pressure as government-led 'big science' projects are questioned.

Under a further proposal for administrative reform being considered by the government, nuclear energy research appears likely to be handed over from a defunct STA to the Ministry for International Trade and Industry, which already regulates the nuclear industry (see *Nature* 388, 815; 1997).

Although it is rarely admitted, the ministry has long been opposed to STA's nuclear fuel cycle policy. At a recent meeting of the committee on fast-breeder reactors, Tomihiro Tominaga, a high-ranking ministry adviser, was reported as having said that there was no need to rush with fast-breeder research.

Robert Triendl

World's biggest synchrotron open for business

[TOKYO] SPring-8, the world's largest third-generation synchrotron radiation facility, began operation last week at Harima Science Garden City in Hyogo prefecture, west of Osaka, Japan.

The ¥130 billion (US\$1.1 billion) facility was planned by the Science and Technology Agency (STA) in the late 1980s in a bid to push Japan to the forefront of synchrotron technology.

Construction, which took six years, was overseen jointly by STA's Japan Atomic Energy Research Institute (JAERI) and the Institute of Physical and Chemical Research (RIKEN), but SPring-8 will be run by the newly formed Japan Synchrotron Radiation Research Institute.

The 8-GeV synchrotron is now the most powerful in the world, surpassing the 7-GeV Advanced Photon Source at Argonne National Laboratory in the United States and the 6-GeV European Synchrotron Radiation Facility (ESRF) at



Charmed circle: SPring-8 is a year ahead of schedule.

Grenoble in France.

The facility is expected to foster a wide range of studies including research on nuclear resonant scattering, structural analysis of matter in extreme conditions and topography and microtomography imaging of crystalline structure. But the 8-GeV synchrotron's advanced features will be particularly useful for analysing protein structures.

"There is no doubt that SPring-8 is the most advanced synchrotron radiation facility around," says Michael Wolff, a beamline scientist from ESRF, who was involved in setting up one of the beamlines at SPring-8. "But there seemed to be little

discussion on the scientific scope of the beamlines, and it will take a good three years to find out whether SPring-8 has a clear advantage over other synchrotron facilities in terms of its technology."

SPring-8 has been commissioned a year ahead of schedule, and has already set up 18 beamlines: 10 for use by outside researchers, six for JAERI-RIKEN projects and the others for machine study. There will eventually be 61 beamlines, including contract beamlines set up by industry and institutions.

One drawback of the accelerated schedule is that it has made it difficult to recruit foreign researchers intended to form a large international users' group. "We have been unable to attract enough foreign researchers this year, simply because we haven't had time to advertise our facility abroad," says Hiromichi Kamitsubo, the director of SPring-8.

Asako Saegusa

Islanders contest report on nuclear risks

[LONDON] A war of words has broken out between politicians on the island of Guernsey in the English Channel and officials at Britain's National Radiological Protection Board (NRPB) over the board's report on the risks to the island from two French nuclear facilities 15 miles away.

The report concludes that radiation doses to islanders from nuclear reactors at Flamanville and a fuel reprocessing plant at Cap de la Hague are not large enough to justify emergency countermeasures.

But many members of the island's 57-strong parliament — known as the States — supported by environmentalist groups such as Greenpeace, say that the report's content does not support such a conclusion.

Drawing on comments from an independent peer review, they claim the report is poorly researched and relies excessively on data from the French nuclear industry. They claim that it fails to address the implications for the island's 60,000 population of a 'worst-case scenario', such as a Chernobyl-style nuclear accident, only assessing smaller scale accidental releases of radioactivity.

One of the external reviewers, Philip Day, reader in chemistry at the University of Manchester, says the report is "seriously hampered" by lack of data. He adds: "Clearly if only trivial accidents are considered, then only trivial results are likely to ensue." He also

raises "doubts as to the accuracy and completeness of the discharge data", claiming the omission of important radionuclides.

Last week, States members invited the NRPB to explain its research methodology at a public meeting. Some members, such as Roland Ogier, want the NRPB to commission new research based on independent data sources, or to reimburse the States the £35,000 (US\$56,000) consultancy fee.

Meanwhile others, such as Jean Pritchard and 19 of her colleagues, have put their names to a letter to Dominique Voynet, the French environment minister, asking for reassurances that the nuclear facilities are safe. "We've waited two years for this report," says Pritchard. "We can't keep having reports if they don't answer our questions."

The prospect of the letter to Voynet is thought to have made the British government nervous, as Britain does not want to be seen interfering with or criticizing France's nuclear activities. Pritchard acknowledges that the letter may land the signatories in trouble as Channel Island politicians are not allowed to deal directly with the French authorities. All official communication takes place through the UK Home Office.

The NRPB report was published at the end of last month. It was commissioned two years ago to assess the safety claims of COGEMA, which runs the fuel reprocessing

facility at Cap de la Hague. The company had told the States in 1995 that there were no significant risks of radioactive contamination beyond a five-kilometre radius of Cap de la Hague, a conclusion that is effectively confirmed by the NRPB.

The NRPB is standing by its document. Stephanie Haywood, the report's editor, acknowledges that the report relies on data from COGEMA on accidental release of radiation. But she says that the data conform to standards laid down by the European Atomic Energy Agency, Euratom.

In the report, Haywood and colleagues included an assessment of radiation doses to the Guernsey population from everyday atmospheric release, from the marine transport of waste, as well as from three potential scenarios of accidental release.

Haywood adds that the criticism that NRPB did not conduct original research on accidental release is unjustified, as the nature and scope of the study had been agreed with the States. She says that States members had been made aware "from the beginning" that the NRPB's expertise was limited to assessing the health risks of radiation. She says a Chernobyl-style accident was not part of the study as the NRPB is not qualified to assess the probability and implications of a major nuclear accident, and that such information has not been released by the site operator.

Ogier, who sent the report for external peer review, says he is "baffled" by such an admission. It appears, he says, that the NRPB has used COGEMA to investigate the safety claims made by that very source.

"I may not be a scientist," he says, "but it is not difficult to tell that the NRPB did not even try and ask the right questions. Some of the report is just O-level physics. Other parts lack a proper analysis and discussion."

The NRPB is supported by the island's Civil Defence Committee, a group comprising members of the States as well as other island officials, which is responsible for advising on nuclear safety. John Langlois, the committee's president and a member of the States, says that if the data supplied are acceptable to institutions such as Euratom, they should be good enough for members of the States of Guernsey.

Ogier says that the issue is of wider significance. "In the past, we've had a very patrician attitude to government — though this is slowly changing." In public, he says, the official line on issues such as the nuclear facilities has always been that there is no risk. "This is done to safeguard interests such as the tourist industry." But, behind the scenes, Ogier says officials are working furiously to establish the truth. Now "there needs to be some admission that there are grey areas." **Ehsan Masood**

Brazil to sequence 'first plant pathogen'

[SÃO PAULO] The creation of a network of laboratories in São Paulo state, Brazil, to sequence the complete genome of a microorganism was announced last week by the Foundation for the Support of Research of the State of São Paulo (FAPESP), the state funding agency.

The Organisation for Nucleotide Sequencing and Analysis will first tackle the bacterium *Xylella fastidiosa*, the causal agent of many economically important plant diseases, particularly citrus variegated chlorosis, which poses a major threat to São Paulo's orange cultivation. This is thought to be the first plant pathogen genome to have been sequenced.

Citrus variegated chlorosis, first reported in 1987, has been found only in Brazil and Argentina. São Paulo and Florida are the two most important citrus-growing areas in the world, São Paulo producing 87 per cent of Brazil's — and 30 per cent of the world's — citrus crop. According to FAPESP, the total cost of the project is US\$11.6 million, to be spent over two years. Sequencing completion is predicted by 2000.

Xylella fastidiosa was chosen because sequencing might help in the control of the

pest, with obvious gains to the state's economy. It will also help to forge links between research centres and the private sector, which is contributing to the cost of the project.

The state says that it is keen to create a network of laboratories that will "significantly increase the number of laboratories in the state capable of using modern molecular biology techniques".

The project will be overseen by a five-member steering committee consisting of three international experts in genome sequencing and two researchers from São Paulo state. Two of the experts, André Goffeau of the University of Louvain in Belgium and Steve Oliver of the University of Manchester Institute of Science and Technology, helped to set up the project, and were also involved in the sequencing of the *Saccharomyces cerevisiae* genome.

The committee will select one laboratory to house a bioinformatics centre. Two large central sequencing laboratories will be chosen to generate a large part of the sequence data. These laboratories will also act as training and support centres for other members of the network. **Ricardo Bonalumé**

Vaccine institute treads out a wary path

[SEOUL] The world's first institution devoted to the research and development of vaccines for developing countries was formally established last week in Seoul, South Korea, when the United Nations Development Programme (UNDP) handed over governance of the nascent International Vaccine Institute (IVI) to an independent board of trustees composed of eminent scientists and health officials.

But the new institute, which will be built in a science park on the campus of Seoul National University and is currently in temporary offices at the university, faces a difficult future as it defines a role for itself in the complex political arena of world health care.

The institute was proposed by the UNDP in 1992. It comes under the umbrella of the Children's Vaccine Initiative, a broad coalition of organizations from public, non-government and private sectors with a secretariat at the World Health Organization (WHO) in Geneva that seeks to protect the world's children against infectious diseases.

The Asian institute is the brainchild of Seung-il Shin, an American of Korean descent. Shin played a key role in the late 1980s in introducing affordable hepatitis-B vaccines to the developing world, and felt the rapidly developing economies of Asia could provide a new source of funds and a means for developing affordable vaccines for the developing world.

Seoul was chosen to host the IVI in 1994 after bids from several Asian countries ended in a three-way run-off between South Korea, China and Thailand. But the international agreement to establish the institute took effect only in May, after three signatory countries ratified the agreement.

Opposition

The institute also had to overcome initial opposition from WHO, which saw it as impinging on its own Western Pacific Regional Office in Manila in the Philippines. Some US vaccine manufacturers were also apparently concerned that it might become a commercial competitor.

WHO's opposition abated after three of its officials were appointed to the 16-member board of trustees, including the head of the Western Pacific Regional Office. And one of the new board's first actions last week was to pass an amendment to IVI's constitution explicitly stating that it will not engage in the sale of vaccines, although it may prepare test vaccine lots for evaluation and clinical trials.

Over the next three years, while IVI's facilities are being built, the institute's staff, working with other Asian research centres and with financial backing from the Korean government, UNDP and private vaccine manufacturers, will concentrate on epi-



The Seoul institute is part of an international initiative to protect children against infectious diseases.

demological and burden of disease studies, and on assessing demand for specific vaccines in Asia, as well as conducting vaccine clinical trials and field studies.

Of particular interest as a first target will be *Haemophilus influenzae* type b, a major cause of bacterial meningitis in young children that seems to be more widespread in Asia than previously thought, but for which no reliable epidemiological data are available to allow effective vaccine application.

After the building has been completed in 2000 and around 200 staff recruited, the institute plans to expand its role to include laboratory research on vaccine candidates, production processes and scaled-up research using a small pilot plant, as well as research into vaccine production quality and safety.

Funding for the first few years of operation is fairly secure. Construction costs for the institute, which could be as high as US\$50 million, will be covered by the Korean government, and of the estimated \$3.9 million operating budget for 1998, the government will provide \$1.5 million, UNDP \$1.0 million and private industry \$0.8 million, leaving only \$0.6 million to be raised.

But when the institute is at full-scale operation and requires an annual operating budget of about \$15–20 million, it is not clear where the money will come from. The Korean government has guaranteed to provide 30 per cent, but UNDP has not guaranteed the rest.

WHO officials on the board are still concerned that the institute could end up tapping into the \$30-million donor budget for the Children's Vaccine Initiative, and institute officials have had to tread warily in defining the institute's role so as not to appear to overlap with the initiative or other WHO activities in the region.

But Richard Mahoney, director of insti-

tutional development at the institute's temporary headquarters in Seoul, argues that the institute is a "new resource that will attract new funds", as witnessed by the substantial contribution from the Korean government and the contributions from Western vaccine manufacturers.

There has been a change of attitude by private industry in recent years, says Roy Widdus, coordinator of the Children's Vaccine Initiative, and Western companies now see a viable market in certain sectors of the developing world and are more willing to work with nonprofit organizations.

Critical

But steady support in the form of donations from countries, in particular the United States and Japan, which have yet to clarify their position on the IVI, will be "critical" for the institute's future, says one board member.

The first meeting last week of a support council for the institute composed of representatives of the 28 signatory nations, prospective signatory countries, donor institutions and industry, attracted a large number of participants. This suggests there is broad support for the institute and countries may be willing to donate new money to it.

Another challenge facing the IVI is recruitment of a top-quality director to head it, a process initiated last week by the board. Seoul does not offer the cosmopolitan attractions of other Asian cities such as Hong Kong and Singapore (which were early candidates to host the institute). Nor is Seoul in a developing country where the director would be seen to be taking on a humanitarian mission. Nevertheless, one institute official says that high-quality individuals have already expressed interest in the post.

David Swinbanks

KENT GILBERT STRINGER/AP

Council of Europe urged to ban human cloning

[PARIS] Political leaders of the 40 member countries of the Council of Europe have called unanimously for a ban on "all use of cloning techniques aimed at creating genetically identical human beings".

The leaders instructed the organization's committee of ministers to adopt quickly a protocol to this effect within its 'convention for the protection of human rights and dignity with regard to the application of biology and medicine', more commonly known as the European Convention on Bioethics, which has already been signed by 22 countries.

The promise to ban human cloning emerged from a summit in Strasbourg last weekend, and was one of a series of statements aimed at updating the Council of Europe's commitment to promoting human rights and democracy.

The cloning ban found itself alongside pledges to fight money laundering, organized crime, racism, corruption, xenophobia and anti-Semitism, and to abolish the death penalty.

Helmut Kohl, the German Chancellor, told the summit that the issue of human cloning was "the greatest moral challenge in

Europe". Germany takes such ethical issues "extremely seriously" said Kohl, referring to the "dark page in our history" (see Briefing, page 660).

The UK Prime Minister, Tony Blair, also championed the ban, declaring that while human cloning was "until very recently the stuff of science fiction fantasy, now it is an all-too-real possibility".

Tax office blocks Russian institute's bank accounts

[MOSCOW] Researchers at the High-Energy Physics Institute in Protvino have appealed to Russian vice-premier, Boris Nemtsov, himself a former physicist, about a decision by the local tax office to block the institute's bank accounts until it has paid its local taxes.

The head of the tax office, Vladimir Kondrashov, has written an article in a local newspaper under the title "Everyone is equal in the face of law", saying that the institute is displaying "a privileged attitude" by withholding taxes.

Researchers at the institute have persuaded the mayor of Protvino, Yuri Ilyin, to write to Alexander Pochinok, the head of the state tax office, asking him to dismiss Kondrashov. So far, Pochinok, who previously worked for 10 years in the Urals branch of the Russian Academy of Sciences Pochinok, has not replied.

Kahn leaves Cochin for Rhône-Poulenc

[PARIS] Axel Kahn, the prominent French medical geneticist who is currently director of the Cochin Institute of Molecular Genetics in Paris, is to join Rhône-Poulenc as deputy scientific director for health and agricultural biotechnology. Kahn has had a long career as one of the country's most prominent figures in the public debate about ethical and regulatory issues associated with new technology.

Kahn is a member of France's national bioethics commission, and was chairman of the committee that advises the government on genetically modified organisms from 1988 until earlier this year, when he resigned following a row with the government over its decision to ban cultivation of a transgenic maize (see *Nature* 385, 667; 1997). His new job will involve evaluating research programmes and strategy in health and agricultural biotechnology throughout the Rhône-Poulenc group.

New head for physics research council

[LONDON] Ian Halliday, currently head of the physics department at the University of Wales, Swansea, is to take over from Ken Pounds as chief executive of the United

Kingdom's Particle Physics and Astronomy Research Council (PPARC). Halliday says he is keen to see increased support for UK physicists, and to increase the prioritization of PPARC research. He has been a member of the PPARC council since 1994.

Kick-boxer jailed for death of geophysicist

[SAN DIEGO] A professional kick-boxer has been jailed for a minimum of 25 years for the murder in 1995 of the prominent British geophysicist, Keith Runcorn, widely known for his work on continental drift. Runcorn, an early proponent of plate tectonics, was killed in a hotel room in San Diego, California, while on a lecture tour (see *Nature* 378, 657; 1995). A former head of the department of physics at the University of Newcastle upon Tyne, Runcorn later became a faculty member of the University of Fairbanks in Alaska. Paul Cain is thought to have killed Runcorn after stealing his wallet and credit cards.

Million-dollar grant for marijuana study

[WASHINGTON] Two months after a panel of external advisers urged the US National Institutes of Health to facilitate studies of smoked marijuana as therapy (see *Nature*

388, 613; 1997), the NIH has granted \$1 million for a study of the smoked drug in AIDS patients. The grant, to Donald Abrams, a professor of medicine at the University of San Francisco AIDS Program, was announced last week.

Abrams and his team will compare the effects of smoked marijuana in HIV-infected patients against a placebo group and against others who take an oral tablet containing THC, marijuana's active ingredient. The researchers will assess the safety of the smoked drug, its interaction with protease inhibitors and its effects on weight gain and appetite. The \$986,000 grant marks a departure for NIH, which, as the only legal source of smoked marijuana for research, had limited funding to studies of deleterious metabolic effects of the drug.

Consumers unhappy with food regulations

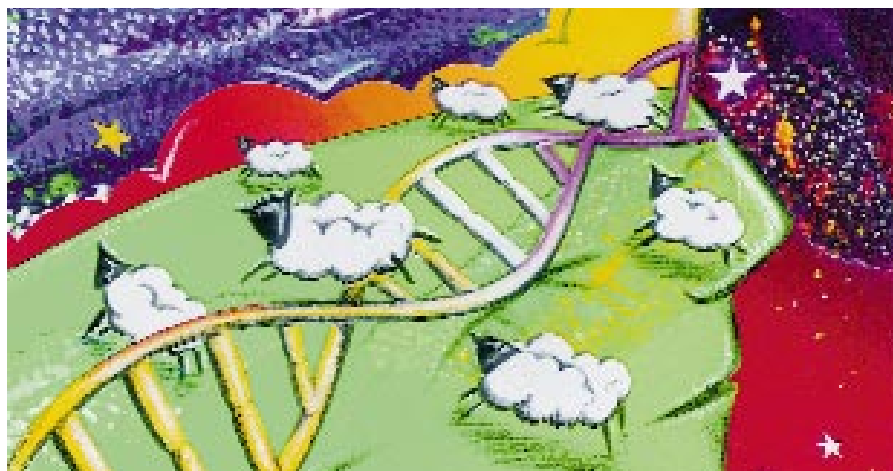
[LONDON] Current regulations governing the approval and marketing of genetically modified foods have failed to satisfy consumer concern, according to a report published this week by the UK Consumers' Association. The report, *Gene Cuisine*, says that a "new approach" to regulating genetically modified foods is needed that can address "the consumer principles of safety, choice, information, and redress".

The report calls for a uniform approach to risk assessment in national, regional and international legislation, and standardized labelling for genetically modified food. It also urges consumer and other organizations to continue lobbying for such food to be segregated from unmodified crops.

Dream date for Ig Nobellists and friends

[BOSTON] The 'Seventh First Annual' Ig Nobel Prize Ceremony, held at Harvard University last week, celebrated the Big Bang, featured a Win-a-Date-with-a-Nobel-Laureate contest and an auction of the plaster casts of the feet of three laureates, and offered the rare chance to hear four participating laureates — Dudley Herschbach, William Lipscomb, Richard Roberts and Robert Wilson — sing their own version of '99 Bottles of Beer on the Wall'. During a 30-second lecture, Lipscomb noted that "if you are not confused by the [Heisenberg] Uncertainty Principle, you do not understand it".

Ig Nobel prizes were awarded in 10 fields, honouring researchers, for example, who measured people's brainwave patterns while they chewed different flavours of gum, citing the discovery that "listening to elevator Muzak stimulates immunoglobulin A production, and thus may help prevent the common cold".



Business booms for guides to biology's moral maze

The past decade has seen the rapid growth of bioethics as a focal point of public concern over new technologies. *Nature's* correspondents report on the ways in which the bioethics movement has developed around the world.

Three years ago, a small biotechnology company inhabited the third floor of a nondescript building in central Philadelphia, on the edge of a major health sciences research campus. Today, a buzzing new business has taken over the space: the University of Pennsylvania Center for Bioethics.

In many ways, the centre epitomizes the emergence of bioethics in the United States both as a public and professional phenomenon. Cloistered 30 years ago in university departments of theology and philosophy, bioethicists today teach overflowing courses throughout universities, inhabit an abundance of institutes devoted solely to their field, and boast a growing media presence.

But, despite their growing prominence, it is far from clear whether US bioethicists have substantially shaped either the culture of science or the political decisions of recent years. "Bioethics has a lot of authority but no real power," says Arthur Caplan, a philosopher with a master's degree in evolutionary biology, who has directed the Pennsylvania centre since its founding in 1994. "We are the proverbial mosquito biting a large elephant."

The numbers of bioethicists — and their output — are growing rapidly. The American Association of Bioethics, founded in 1993, has doubled its membership since 1995. A bioethics literature which in 1966 generated 314 Medline citations last year contained over 3,400 (see right).

"There's a giant literature now which you can identify as bioethics literature," says George Annas, a lawyer who teaches bioethics in the School of Public Health at Boston University. "Everybody before would identify it as a theological literature."

Bioethics "has moved from a pipe dream to a fad to acceptance to the mainstream," says Caplan. The Pennsylvania centre employs 17 philosophers, social scientists, doctors and lawyers who find themselves engaged as much in teaching, shaping policy and educating the public as in writing papers.

Their role has become big business. This year, the centre launched a master's degree programme which received four times more applicants than it could accommodate. 'Hits' on the centre's Internet website have climbed

from 100 a month in 1994 to 30,000–60,000 a month today. Such exposure has generated its rewards. The centre has just clinched funds that will double its \$2 million budget.

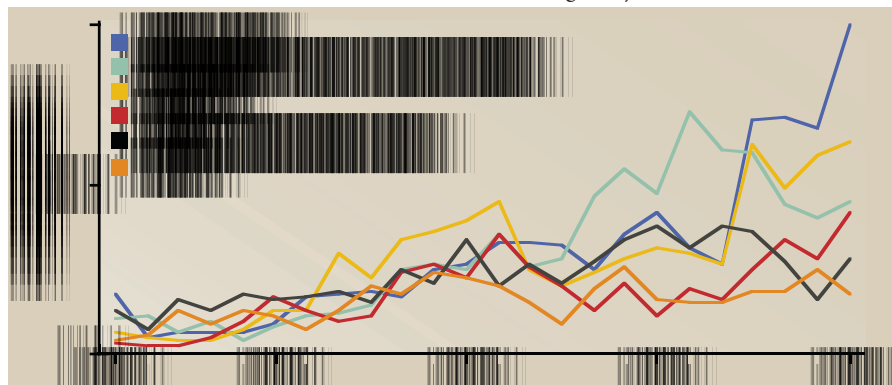
Some say that the media-friendly Caplan, who has a knack for dispensing pithy 'sound-bite' comments — and has been snubbed by some philosophers as a popularizer — is as responsible as any single person for the field's rising profile. But the new prominence of bioethicists can also be traced to the 1988 launch of the Human Genome Programme under James Watson at the National Institutes of Health, and, earlier, at the Department of Energy.

At Watson's suggestion, 3 to 5 per cent of the project's research funds were dedicated to the study of the ethical, legal and social implications (ELSI) of mapping the genome. ELSI funding reached about \$9 million in 1997. Caplan has dubbed it "a full employment programme for bioethicists".

Armed with the conclusions of such government-funded studies, bioethicists have claimed seats on government panels advising on issues from blood safety and embryo research to President Bill Clinton's ill-fated health reform plan. Media coverage has also played a role: since February, Clinton's National Bioethics Advisory Commission (NBAC) has vaulted to prominence largely on the wave of concern over the cloning of Dolly the sheep (see *Nature* 386, 644; 1997).

But questions remain about the extent to which bioethicists have influenced science and politics. Some bioethicists bemoan their lack of impact on the education of bench scientists. In contrast to their success in integrating bioethics into medical school curricula, "the research ethics end of it has been quite the failure," says Mildred Cho, a biologist who works at the Pennsylvania centre.

Bioethics education for federally-funded student scientists, she says, consists of pulling students into a room for a few hours, then sending them back to the culture of the laboratory. David Asch, a physician at the centre, adds: "You don't have to be too cynical to realize that [such training] is probably not having a major effect."



The rapid rise in citations to ethics in six leading journals.

In the political arena, the relatively liberal recommendations of bioethicists on government panels studying fetal tissue and human embryo research have over the past decade been mostly disregarded by Republican presidents and Congresses. Even Clinton said that embryo research recommendations went too far, by allowing the creation of human embryos for research, when he banned federal funding for the practice. And the sole legislation to emerge from the ELSI programme, a model genetic privacy law, has been largely ignored by lawmakers.

Cloning ban

On cloning, congressional Republicans have rapidly shunned the NBAC, drafting — in the only bill to pass a committee so far — a far more conservative prohibition than the commission recommended (see *Nature* 388, 505; 1997). Indeed, apart from a couple of early, significant, victories, “it’s hard to show any concrete influence” on policy by US bioethicists, says Boston University’s Annas.

Part of the reason stems from the lack of political influence of bioethicists in a society that traditionally tries to resolve issues by lawmaking rather than by agreeing common ethical standards of behaviour. “A tendency of American bioethics is to shift the debate from whether something is right or wrong to whether it should be outlawed or not,” says Annas, who contends that bioethics should move its focus “beyond defining the minimum morality the law requires and more into the realm of the right and the good”.

Advocates with strong opinions on bioethical issues agree with him. They were dissatisfied, for instance, with the NBAC’s recommendation last spring for a five-year legal moratorium on cloning for reproduction on the basis of safety concerns. NBAC postponed religious and ethical questions, saying they should be dealt with in a broad societal debate before the moratorium ends.

This drew criticism from conservatives, who desired an outright declaration of cloning’s immorality. At the same time, critics on the left complained that the NBAC ducked questions such as why asexual as opposed to sexual reproduction should be an affront to the dignity of the species.

More importantly, says Annas, the strictly legal approach to US bioethical decisions has resulted in one particular “cloud” over US bioethics. In 1973, the Supreme Court determined that abortion is legal before fetal viability, leaving private individuals to decide on its morality. But the lack of a US moral consensus on abortion has meant that issues from human embryo research to fetal tissue transplants to cloning remain bedevilled by the subtext of abortion politics.

Still, despite recent setbacks, some early victories of US bioethics remain significant.

Russia warned: act now or regret it later

If the Russian government fails to introduce legislation on genetic engineering soon, “in two or three years it will be too late — the state will not be able to control the safety of newly produced food and medicines”. That was the warning given last month by Rem Petrov, the Russian Academy of Sciences vice-president for biological sciences.

Petrov was speaking at a symposium on assessing the safety of genetically modified crop plants and novel foods on the Russian market. The meeting was organized by the academy and the United Nations Food and Agriculture Organization.

Amirkhan Amirkhanov, deputy chairman of the state committee on the protection of the environment, said that the rapidly growing number of small private companies experimenting with plant, animal and even human genes increased the need to introduce regulations as rapidly as possible. But a major obstacle was in persuading officials of the need for action.

Perhaps significantly, the strongest voice heard at the meeting in favour of the tight regulation of genetic engineering represented neither researchers nor the government, but private industry.

Yuri Kalinin, general director of the Biopreparat company, which makes more than 40 per cent of Russia’s biotechnology products, said his enthusiasm for regulation was based on the difficulties he had experienced with foreign partners. Their goods and biological components were required to pass various examinations but, even though they had received international or national safety certificates, these were not valid in Russia owing to lack of legislation.

Konstantin Skryabin, director of the academy’s Bioengineering Centre, which hosted the meeting, said that some of the laws sought by the scientists and businessmen have already passed through the State Duma, the lower chamber of the Russian parliament.

Carl Levitin

The most important, most agree, was the adoption of government protections for human research subjects. These were informed by the earlier drafting of the Nuremberg Code and shaped by the National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research. The commission was set up in response to the 1972 revelation that the US government had for 40 years funded the notorious Tuskegee Syphilis Study, in which treatment was withheld from 399 black men with the lethal disease.

Model law

A later commission, the President’s Commission for the Study of Ethical Problems in Medicine and Biomedical and Behavioral Research, launched in 1979, laid out a model law on the determination of death that has been adopted by a majority of states. Separately, a *de facto* prohibition on the use of anencephalic infants as heart donors “was virtually single-handedly a victory of bioethicists”, says Paul Root Wolpe, a sociologist at the Pennsylvania centre.

But today, the government’s interest in soliciting bioethical opinion may reflect less the influence of such opinion than the fact that the public displays a seemingly insatiable appetite for bioethics ‘experts’ to analyse the social significance of biomedical developments. In the two months after the announcement that Scottish scientists had cloned Dolly, for example, the Pennsylvania centre’s website drew one million hits. Clinton’s reaction to Dolly was to ask the NBAC — which until then had languished, under-

funded, in obscurity — to produce policy recommendations within 90 days.

Some argue that this leads bioethicists into perilous waters, as the government can quickly exploit their conclusions to provide cover for difficult or unpopular political decisions.

“The irony of ethics is that it tends to be scandal driven,” says Wolpe, suggesting that what he calls “crisis intervention bioethics” may not be the most effective route to rational policy.

The problem is exacerbated for government-funded ethicists, who, Annas contends, become hostage to their funders and are forced into dealing with “very, very narrow parochial interests... within a very short time frame.” There may be further peril in the private sector, whose increasing wooing of bioethicists risks turning them into “a domesticated species”, says LeRoy Walters, director of the Kennedy Institute of Ethics at Georgetown University in Washington DC.

Two pharmaceutical companies, Smith-Kline Beecham (SKB) and Zeneca, are paying for employees enrolled in the Pennsylvania master’s programme. SKB also donated almost \$1 million in 1995 to Stanford University to fund research on genomics, ethics and society. And the American Medical Association has launched an Ethics Institute.

Both business and government will soon have more than their pick of bioethicists. Students are packing the many bioethics courses that have appeared over the past decade. Michael Grodin, director of bioethics at Boston University, says student interest “is booming”.

Meredith Wadman

Germany's past still casts a long shadow

Germany's approach to bioethics has been hesitant and uncoordinated — a fact that should surprise no-one. For debates on whether such a discipline exists separately from ethics, whether Germany should ratify the Council of Europe's Convention on Human Rights and Biomedicine, and even whether it should set up a central bioethics committee, still rage with an intensity unknown elsewhere.

Inevitably, German attitudes remain influenced both by guilt over the abuse of medical research in the Nazi era, and by the so-called Nuremberg code, which was drafted in 1947 to ensure that experiments on non-consenting humans should never happen again. Despite this code, many Germans fear that any venture into the realm of genetics could reopen the door to the abuses of the Nazi era — a fear largely responsible for molecular biology's slow start in Germany.

Recently, however, the commercial and medical benefits of molecular biology being enjoyed by its main economic competitors has jolted Germany into making a concerted effort to catch up. Bioethics is not far behind, spurred partly by an awareness that difficult issues need addressing if these benefits are to be enjoyed, and partly by a desire to fall in line with other European nations.

Frequently heated debate on bioethical issues involves a wide range of church groups, environmentalists and patients' rights groups. Much of the efforts of these groups is presently focused on the Council of Europe's Convention on Human Rights and Biomedicine, which was approved by its parliament in April.

Germany has not yet signed the convention because of a clause allowing research on the brain to be carried out on individuals incapable of giving consent, which many feel undermines the constitutionally 'inviolable' human dignity, and another clause allowing a limited amount of embryo research.

Bringing round the churches

The German government, keen to fit in with the rest of Europe, has negotiated long and hard with the most influential opponents of the convention, including the churches. While neither of the two main churches positively support the convention, the Roman Catholic church has now agreed not to actively oppose signing, and the protestant church is maintaining a neutral stand. The government will probably sign before the end of the year.

But it remains uncertain whether parliament would ratify the convention. The two main parties are split on the issue, while the Greens are unanimously opposed, arguing that signing could open the door to abuse. The Greens were among the many pressure



groups that persuaded the Council of Europe to change the name of the convention from its working title of 'bioethics' to 'human rights and biomedicine'.

The intensity of the debate over the convention reflects the emergence of a bioethics movement. This has had to graft itself on to the traditional system of medical ethics. This system takes as its fundamental principle the first article of the German post-war constitution — that "the dignity of humans is inviolable" — and has until recently remained firmly in the hands of physicians.

"We used to simply follow the advice of the German Chamber of Physicians," says Ludwig Honnefelder, director of the Bonn Institute for Science and Ethics, and one of Germany's few bioethics specialists. "Even now genetic counselling is only carried out by physicians alone, not with the advice of biologists as is common elsewhere."

In the 1980s, it was already clear to politicians that some of the issues raised by developments in biology had become too complicated for physicians to solve on their own. In 1984, the ministries of research and legal affairs set up an ad-hoc committee to make recommendations for legislation, particularly in the areas of *in vitro* fertilization and genetic analysis and gene therapy.

A second commission issued a set of recommendations relating to genetic engineering in 1987. This body was called for by the German parliament and headed by Munich-based molecular biologist Ernst-Ludwig Winnacker, currently the president of Germany's main research grants council, the Deutsche Forschungsgemeinschaft (see *Nature* 388, 507; 1997). A third committee, set up by the federal and Länder (state) governments to consider ethics and reproductive medicine, reported in 1988.

These reports eventually led to legislative decisions more restrictive than most scientists serving on the committees would have liked, because of the moral concerns of some parliamentarians. The 1991 law on protection of the human embryo forbids any form

of embryo research, and the 1990 gene laws were so restrictive that they were modified in 1994 (see *Nature* 359, 93; 1992).

Germany's approach to bioethics problems continues on this ad-hoc basis. So the cloning of the sheep Dolly caught the government unprepared. Research minister Jürgen Rüttgers asked seven scientists, led by Hubert Markl, president of the Max Planck Society, to produce an immediate position paper on human cloning.

Need for a forum

But the Dolly affair brought home to Rüttgers the need for an established forum to give guidance on bioethical issues. He promised to "consider what to do to strengthen the position of applied ethics in science". A spokesman for the ministry makes clear that Rüttgers does not intend to create a central committee, however, preferring to tap expertise in Germany's half-dozen or so ethics institutes.

This reluctance is partly linked to Germany's federal structure, and partly to the fear of scientists and the research ministry that bioethics could become over-regulated. Scientists such as Markl and Detlev Ganten, director of the Max Delbrück Centre for Molecular Medicine in Berlin, believe the Central Committee on Ethics of the Chamber of Physicians remains sufficient for consideration of ethical issues at federal level.

But ethicists such as Honnefelder believe that an independent federal ethics committee is now needed, both to raise the status of bioethics in the scientific community and to nominate a national representative to attend the regular meetings between the bioethics committees of all European Union states.

Details of a proposed national bioethics advisory group, similar to that in France and elsewhere in the union, are being developed by the opposition Social Democrat party. "We have a lively discussion about bioethics in Germany," says Wolf-Michael Catenhusen, secretary of the party's parliamentary group, "but no focus for it." **Alison Abbott**

France reaps benefits and costs of going by the book

A stunt show in which a 'flying dwarf' had been projected from a cannon was recently shut down in France on the grounds that it contravened 'human dignity', a ruling which might well bewilder other nationalities, Anglo-Saxons in particular. (An appeal by the dwarf to the European Court of Justice, on the basis that the decision infringed his 'dignity' to work, was thrown out on technical grounds).

In the same way, France's abstract approach to bioethics, relying on a battery of laws peppered with references to universal principles such as 'human dignity' and 'human rights', often baffles observers from countries, including the United States, with little or no bioethics legislation. Indeed, the French philosophy on bioethics is sharply opposed to the 'pragmatic' approach found in both the United States and the United Kingdom, which tends to address specific bioethical problems in an ad-hoc fashion.

Nowhere has this difference in philosophy been seen more clearly than in the exchanges over the ethics of cloning (see *Nature* 385, 810; 387, 754; 388, 320 & 511; & 389, 433; 1997). Arguments by Axel Kahn, a member of the French national bioethics committee, that cloning is an affront to human dignity have been dismissed as "rhetoric" by two leading British bioethicists.

David Shapiro, former executive director of the UK Nuffield Foundation on Bioethics, says that the debate should focus on whether cloning is ethical in tangible cases, such as in men unable to reproduce sexually. But Kahn says that such arguments are typical of the "utilitarian" approach endemic in Britain and other Anglo-Saxon countries, which reduces ethical problems to an "algebraic equation of the pros and cons" of a particular situation. "If the pros exceed the cons, then it is judged ethically acceptable," says Kahn.

The Anglo-Saxon tendency is to rely on

professional codes of conduct as the main means of regulation (see page 663). In contrast, France has invented a highly institutionalized system for regulating biomedical progress that reflects the philosophy by which it is legitimized.

Indeed, as far as government action is concerned, France has been in the vanguard of the bioethics movement. In 1983, for example, it became the first country to create a national bioethics committee, and the world's first comprehensive bioethics legislation was introduced a decade later.

International influence

Similarly, France has had a major influence in the drafting of the Council of Europe's Convention on Human Rights and Biomedicine, and the proposed 'universal declaration on the human genome and human rights', due to be approved later this year by the United Nations Educational, Scientific and Cultural Organization (Unesco).

Nöelle Lenoir, a member of the French constitutional court, who played a major role in shaping the country's bioethics legislation, is chairwoman of Unesco's International Bioethics Commission, and of the

Japan's bioethics debate lags behind thinking in the West

The word 'bioethics' — *seimei rinri* (life ethics) — is familiar to most Japanese people, but its use tends to be different from that in the West. Issues such as 'brain death' and organ transplants have generated widespread concern. But there is little active public discussion of the ethical implications of techniques such as genetics and cloning.

The political difficulty in confronting the ethical dilemmas raised by new biomedical techniques was reflected in the debate over

accepting 'brain death' as death, triggered by Japan's first and only heart transplant, performed in 1968. Still referred to as the 'Wada transplant' after the surgeon who carried it out, the operation ended in controversy

when the patient died and the medical team was accused of murder.

One result was that organ transplantation was put on hold. Only now, nearly 30 years later and after heated debate, has Japan finally passed a bill allowing the transplant of organs from brain-dead donors (see *Nature* 387, 835; 1997). Meanwhile, guidelines or regulations for cloning or handling of human genes have yet to be

developed by the government, reflecting low public concern on such issues.

Darryl Macer of the University of Tsukuba, who runs the Eubios Ethics Institute, says one apparent explanation is that policy makers "are more concerned with promoting public acceptance than exploring ethical issues in decision making". Macer points out that Japan is a paternalistic society, where the views and opinions of 'experts' are usually followed uncritically.

A survey this year, led by Eubios Ethics Institute, revealed that the news about the cloning of the sheep Dolly had made little public impact in Japan. Indeed, the survey revealed that 30 per cent of those interviewed were not aware of research in genetic technologies, while another 30 per cent were generally supportive of such research. The rest felt unable to choose between the risk or the benefit of genetic technologies.

Norio Fujiki, emeritus professor at Fukui Medical School, says the survey showed that the Japanese people are generally supportive of genetic technologies and scientific research, even though many are poorly informed on the subject. "People still find genetics 'mysterious', and have biased and misleading ideas," says Fujiki.

In principle, the Japanese people say that they are supportive of genetic screening — as well as of gene therapy. But screening services are not readily available, and genetic counselling is not widely practised. Indeed, under the 1948 Eugenic Protection Act,

which was abolished in 1995, it was illegal to abort fetuses which had genetic diseases and chromosomal abnormalities. Fujiki, who has been providing genetic counselling in Japan for 35 years, says that people still have preconceived ideas about genetic diseases which they are reluctant to discuss. Many view the lack of debate on such topics — and the delay in reaching a consensus on the need for government action — as stemming from Japan's cultural and religious background.

Some sociologists and religious groups disagree with this interpretation, saying opposition to new biomedical technologies is based merely on a lack of understanding. But the government appears relatively unconcerned about bioethical issues.

Admittedly some efforts have been made by the government to integrate ethics into decisions about gene therapy and brain death. The Science and Technology Agency (STA), which is setting up a genome research centre in the Institute for Physical and Chemical Research (RIKEN), is planning to set up a bioethics section to investigate bioethical issues arising from the life science research programmes run by the agency.

But many are still calling for proper guidelines, and for proper education in bioethics. Even the STA's plan has only been given ¥8 million (US\$65,000) funding, and some are concerned that it could end up as a technology assessment committee, rather than a body that monitors ethical issues involved in the research.

Asako Saegusa



Unlike in Japan, the cloning issue has generated widespread protest in Korea.

YUN JAL-HYOUNG/AP

UK takes pride in 'principled pragmatism'

Early last year, the British government announced that it had decided not to follow the advice of a committee of the House of Commons to set up a panel to monitor the social impact of human genetics research, arguing that this would create unnecessary bureaucracy (see *Nature* 379, 195; 1996).

But within two months the government had changed its mind, announcing plans to create a Human Genetics Advisory Commission which would take a "broad perspective on the implications of genetics".

Members of the committee prided themselves that the government had eventually accepted the logic of their case. A key factor was that senior politicians appear to have been made aware that action was needed to contain public disquiet over the way a number of critical issues raised by genetics had been handled, and to provide reassurance about such issues in future.

The pattern is a traditional one in the United Kingdom. A strong distrust of central authority — contrasting, for example, with the French acceptance of a powerful state — has discouraged an excessively legislative approach to ethical issues. Where action has been taken, it tends to be reactive rather than proactive. That gives flexibility, but opens decisions to political pressures.

Britain's preference for what one ethicist calls "principled pragmatism" has favoured greater reliance on professional codes of conduct and voluntary guidelines with minimal legislative backing, and ad-hoc commissions of inquiry into specific topics — such as the use of fetal tissue or, most recently, commercial genetic screening kits.

But there has also been a growing awareness in recent years of the need for a more institutional approach to the ethical issues raised by developments in the biomedical sciences, backed by a desire to ensure that public concern over these issues does not become too disruptive.

Growing concern

There is plenty of evidence that this concern is growing. It has been spurred not only by individual scientific developments — the most obvious being the cloning of the sheep Dolly at the Roslin Institute in Scotland — but also, some argue, by deeper and less well articulated fears about modern science.

According to Martin Bauer, for example, a sociologist at the London School of Economics, analysis of media coverage of stories on topics such as genetics reveals a significant increase in the emphasis given to the moral rather than safety dimensions of their likely impact. "Risk as such appears to be becoming less relevant," says Bauer, referring as an example to debates over genetically-modified foods. "What people seem to be

interested in is whether a particular scientific development is part of the natural order, or whether a moral order is being transgressed."

The strength of this feeling has often taken the government — and the biotechnology industry — by surprise. As elsewhere in Europe, for example, one factor spurring a new political interest in bioethics was the unexpected rejection by the European Parliament two years ago of a bid to harmonize biotechnology patents throughout the European Union (see *Nature* 374, 103; 1995).

Opposition to the move focused on part of the new rules confirming the legal right to patent human genetic material. Following an active lobbying campaign by the industry, the rules have now been passed in a revised form. But memories of the initial vote, which could if sustained have caused major problems for the industry, remain strong.

Training need

One response to such types of situations in the academic world has been greater awareness of the need to include examination of ethical issues in the training of scientists. "Scientists and engineers must be prepared to live in a society in which a whole panoply of new mechanisms is coming into force," says Ray Spier, currently professor of microbiology at the University of Surrey, who next month takes up the country's first chair in science and engineering ethics.

In research funding organizations, there has also been a new willingness to look seriously at the public response to the research they support. The Medical Research Council last year set up an advisory panel to assess the implications of some of its more controversial projects — such as those on the genetic

basis of social behaviour. Similarly the Wellcome Trust, now almost equal in influence to the MRC, is about to announce a major initiative to support work on the ethical and social implications of research ranging from genetics to the neurosciences.

Much recent action on ethics-related issues has been prompted by the Nuffield Council on Bioethics. Set up in 1990 as a focus for debate on issues not already covered by medical bodies such as the Royal College of Physicians, the council has helped mould government thinking on topics from genetic screening to xenotransplantation.

Brian Wynne, professor of science and social policy at the University of Lancaster, argues that the issues raised by bioethicists are often those "secondary risks" which have been marginalized by an excessively reductionist interpretation of risk and safety. "The problem is that there is no language to talk about them in the context of the [conventional] risk debate," he says.

And Ruth Chadwick, who runs the Centre for Professional Ethics at the University of Central Lancashire, says that a broader definition of bioethics is needed, arguing that it has tended to concentrate on the impact of biomedical developments on individuals, to the neglect of its wider social consequences.

The lack of consensus on the role of bioethical arguments in policy debates remains frustrating to some. But others such as Chadwick say that it offers an opportunity to integrate into public policy genuine public concerns about science. Science minister John Battle has offered to boost this process by supporting a 'consensus conference' on genetics through the Human Genetics Advisory Commission.

David Dickson

Policing ethical codes in India proves tough

The ethical dilemmas raised by modern biomedical research are not confined to industrialized countries, and many less developed and industrializing nations are also growing increasingly concerned. India is tightening its ethical guidelines for biomedical research and is setting up a national bioethics advisory panel, although policing such guidelines is likely to remain an uphill task.

In the early 1980s, for example, despite the recent introduction of ethical guidelines for research on human subjects, a trial carried out at one of the institutes of the Indian Council of Medical Research (ICMR) involved withholding treatment from more than 1,000 women to enable researchers to find out if lesions in their uterine cervix turned into cancer.

Eventually more than 70 women

developed cancer and two died during radiation therapy. "Our problem is not writing guidelines but their implementation," says Avtar Singh Paintal, former director general of ICMR and founder president of the Society for Scientific Values, whose goal is to promote ethics in research.

A 15-member committee headed by M. N. Venkatachallaiah, a retired chief justice, is currently revising the ethical guidelines covering biomedical research, taking into account issues raised by modern genetics. Various subcommittees are evolving an ethical code for research in epidemiology, genetics and human tissue transplants.

Another committee is being set up by the Department of Biotechnology to draw up guidelines for human trials of recombinant products.

K. S. Jayaraman

Pre-empting the arrival of a dark lord

Sir — In a recent leading article, “Obstacles of nomenclature”, you scolded certain molecular biologists “whose profligate and undisciplined labelling is hampering communication” (*Nature* **389**, 1; 1997). As our laboratory is responsible for some of the “offending gene names”, I feel compelled to set the record straight.

The names with which I wish to take issue are a group of *Drosophila* genes: *numb*, *prospero*, *inscuteable* (not “*inscrutable*” as you had it) and *miranda*. These genes are involved in asymmetric cell division. The leading article complained that the names of the genes give no indication that they are involved in such functions. The reason is quite simple: when this group of genes was first named, nobody had any inkling that they had anything to do with asymmetric cell division.

Traditionally, a newly discovered *Drosophila* gene is named by the discoverer(s) according to its mutant phenotype noted then. Of this group of genes, the first was discovered in 1989. T. Uemura in our laboratory identified mutant flies that have only about 10 per cent of the normal number of sensory neurons. These mutants presumably receive much reduced sensory input and so we named the gene *numb*. The next one in the group, *prospero*, was named by C. Doe and M. Scott in 1991. One of the phenotypes of *prospero* loss-of-function mutants is a change of cell fate. Doe and Scott decided to name that gene after the main character in Shakespeare’s *The Tempest* because of Prospero’s presumed ability to influence fate. It was not until 1994 that we first realized that *numb* is involved in asymmetric cell division.

Later, the work of several laboratories demonstrated the involvement of *prospero* and *inscuteable* in asymmetric cell division. In the case of *prospero*, it was not difficult to imagine that the segregation of a nuclear protein to a crescent on the cell membrane before cell division is reminiscent of Prospero’s exile to an island. It therefore

seems appropriate to name a protein that associates and localizes Prospero after Miranda, his daughter and companion in exile.

You may then ask why the people in the field didn’t get together and rename the genes to reflect their roles in asymmetric cell division? There are good reasons not to do so.

First, it is an excellent tradition among *Drosophila* workers that once a gene is named the name stays. This tradition has been followed for many decades with only rare exceptions. This allows subsequent workers to pay homage to earlier workers and prevents the possibility of a dark lord coming along and renaming genes, thereby confusing future generations into thinking of him as the discoverer of everything.

Second, an important lesson from modern biology is that the action of a gene is often pleiotropic. A gene is often used at multiple developmental stages for different functions. For example, *prospero* is not only involved in asymmetric cell division but also has an important function in controlling axon growth and guidance. If one were to rename *prospero* to reflect its role in asymmetric cell division, one would ignore the other, equally important, function. Moreover, our knowledge of a gene changes with time. It is not uncommon to discover a completely new and unsuspected function for an old familiar gene. Do we then rename a gene every time we learn something new about it? Of course not. We revise and update the knowledge associated with the gene.

Does the naming system of *Drosophila* genes hamper communication? I don’t think so. Perhaps it is in the nature of human memory that many biologists (not just *Drosophila* workers) find it easier to remember and associate the individual, sometimes whimsical, *Drosophila* gene names with their function than to keep track of the differences between *unc-37* and *unc-39* of *Caenorhabditis elegans* or between *cdc24* and *cdc42* of *Saccharomyces*

cerevisiae even though, in the latter cases, workers in the field have adopted a more systematic naming system. Many of us were glad that C. Nüsslein-Volhard and E. Wieschaus chose whimsical names such as *armadillo* and *hedgehog* (instead of *gap-1*, *pair-rule-2*, *segment polarity-3* and so on) for the genes they discovered in their historical screen for *Drosophila* body patterning mutants. This system works well enough that a similar naming system has been adopted for zebrafish genetics.

Finally, I wish to quibble with two more points in your leading article. You say: “the lack of a common classical education explains today’s avoidance of the archaic but otherwise constructive habit of giving new things names that have a Latin or Greek etymology. The consequence has been a descent into whimsy. Murray Gell-Mann started it all with his quark...”. There are two problems with these statements. Cultural chauvinism notwithstanding (one man’s classical education is another man’s low-level baloney), for the reasons already stated, I don’t see how naming things with Latin or Greek origins would help in the case of *Drosophila* gene names. And it is ironic that you juxtaposed Gell-Mann with “lack of classical education”. Of all the twentieth-century physicists, Gell-Mann is probably the last person one would have thought of as having a “lack of classical education”.

You also say: “Regrettably, molecular biologists have followed the particle physicists’ whimsy with obscurantist enthusiasm.” Don’t blame it on the particle physicists. *Drosophila* geneticists already used whimsical names before “three quarks for muster Gell-Mann or three aces for Mr G. Zweig”.

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There are ‘two cultures’

Sir — David Edgerton is wrong, and C.P. Snow was right (*Nature* **389**, 221; 1997). Every scientist knows people thoroughly versed in literature, music and the arts who are proud not to understand mathematics, physics and chemistry, do not comprehend the scientific process, have no idea of what is going on in the scientific world and are unable to follow the instructions for use of a

video recorder. That science and technology play such an important role in our life is no proof that this abyss does not exist, but shows only that science and technology make our lives easier, and that the economy can grow only by creating new products based on science and technology.

To deny that there are strong antiscientific currents in our society is to shut our eyes to the broad resistance against, for example, necessary animal

experiments, or gene technology for agriculture. In a scientifically minded society, it would be impossible for astrology and other esoteric practices to flourish.

The ‘two cultures’ are not a British phenomenon but can be found equally in other European countries, particularly Germany and Austria.

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Technology and the single electron

Marc Kastner

Since their invention, announced in 1948, transistors have become an irreplaceable part of modern life. How can they be made ever tinier? A marriage of lithography and colloidal chemistry is a promising approach.

The ability to make progressively smaller structures, developed for the electronics industry, has allowed physicists to study how electrons behave when confined to reduced dimensions. Simultaneously, a new approach is being developed, one in which colloidal chemistry makes it possible to build up nanometre-sized structures atom by atom. The integration of such self-assembled nanostructures with lithographically defined electrodes is likely to reveal new physics, and may also lead to new electronic devices based on quantum-mechanical effects. On page 699 of this issue¹, Klein *et al.* report an important step in the merging of these two technologies.

The most exciting discoveries in solid-state physics in the past two decades result from the confinement of electrons. For example, confining electrons to two-dimensional motion in a strong magnetic field creates a new state of matter characterized by the quantum Hall effect. The Hall effect is a voltage that is generated perpendicular to both the current flow and the magnetic field. At low temperature and high field, the ratio of this Hall voltage to the current is accurately quantized. The quantum Hall effect is observed by confining the electrons by man-made material boundaries inside a transistor^{2,3}. Similar devices, called field-effect transistors, or FETs, are found in computers and cellular phones.

These FETs are simple devices consisting of three layers — a metal (called the gate), an insulator and a semiconductor (Fig. 1a). There are two contacts attached to the semiconductor, called the source and drain, that allow electrons to enter and exit, and another contact to the gate. When the voltage on the gate V_g is positive, electrons are pulled into the semiconductor, it becomes conducting, and current can flow in response to a voltage applied between source and drain. When V_g is negative, electrons are removed, the semiconductor is an insulator and no current can flow. Thus the FET is a switch that turns on when electrons are added and off when they are removed.

A new kind of transistor is created when electrons are confined to a small volume of space between the source and drain, separated from the two contacts by thin insulating barriers. These barriers allow the electrons to enter or exit only by quantum-mechanical tunnelling. According to quantum mechanics, electrons can penetrate regions of space from

which they would classically be excluded. This is called tunnelling. Because of the confinement, both the charge and energy of the electrons become quantized, so the small volume behaves like an artificial atom^{4,5}. In general, additional energy is required to add (or subtract) an electron to the confined region; so, for most values of V_g , current cannot flow through the transistor. However, for a specific value of V_g , the energy of the transistor with N confined electrons is equal to that with $N+1$ confined electrons. For this V_g , the charge on the artificial atom can fluctuate and current can flow. This is analogous to the equilibrium between two oxidation states of ions in solution in an electrochemical cell. Because the transistor conducts at one value of V_g for every

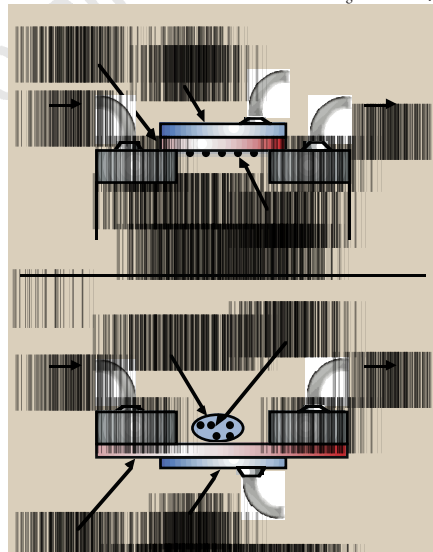


Figure 1 Field-effect and single-electron transistors. a, A field-effect transistor, like that used in computer memories, consists of a semiconductor with metallic source and drain contacts, and a metallic gate electrode nearby, separated from the semiconductor by an insulator. When the voltage on the gate is positive, electrons accumulate in the semiconductor, making it conducting and turning it from the 'off' to the 'on' state. b, A single-electron transistor (SET) is very similar, although the gate is shown on the bottom, as in the device of Klein *et al.*¹, discussed here. The semiconductor is a small particle, separated from source and drain by barriers to quantum-mechanical tunnelling. The SET turns on and off again every time an electron is added to the particle.

value of N , it turns on and off again every time an electron is added to it. That is why it is called a single-electron transistor (SET; Fig. 1b).

SETs have been made in a number of ways using lithographic techniques⁴. But most of them operate only at very low temperatures, typically less than 10 K. The temperature of operation is proportional to the energy required to add an extra electron to the artificial atom; and, because the latter results primarily from the Coulomb repulsion between electrons, it varies as the inverse radius of the confined region. So, to make SETs that operate at higher temperature, one needs to confine electrons to smaller volumes than are accessible with current lithographic techniques.

In the past few years, however, chemists have learned to make volumes of semiconductor as small as 15 Å in diameter using colloidal chemistry⁶. The slow growth of the nanocrystals allows highly precise size selection, so that huge quantities of nearly identical crystals can be produced. These would make wonderful SETs if one could attach leads to them. But, whereas lithography is limited to creating volumes larger than about 500 Å, the nanocrystals are limited to sizes less than about 150 Å; if they are larger, they precipitate out of the colloidal suspension. A great technological challenge is to bridge this size discrepancy.

Klein and co-workers¹ have found a clever way to show that this can indeed be done. They prepare a metal gate with an insulator on top of it, and use lithography to create metal source and drain electrodes on the surface of the insulator. Because only a gap is needed between source and drain, rather than a volume, Klein *et al.* are able to make the gap as small as about 50 Å. When they finally deposit 55-Å semiconductor nanocrystals on the surface, once in a while a single particle falls in the gap, creating a single-electron transistor. This is the first time that a complete SET has been fabricated with a colloiddally grown nanocrystal as the confining region. Furthermore, the energy scale observed is quite large.

In itself, the technique is not likely to produce large numbers of useful devices. Nonetheless, Klein and colleagues' successful demonstration-of-principle suggests that the combination of lithographic and chemical techniques might eventually become a practical means for production of the smallest transistors yet. □

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Developmental neurobiology

Unscrambling a disabled brain

André M. Goffinet

The orderly development of neurons in the mammalian brain is a conundrum that defies imagination: the billions of neurons that are generated around the cerebral ventricles must migrate through the tissue to find their destination, before forming exquisite cell patterns. The formation of these patterns is abnormal in mice with the *reeler* phenotype, showing that it is controlled by the *reeler*, *scrambler* and, probably, several other genes.

Although the mechanisms by which pattern formation is regulated remain largely unknown, the gene that is mutated in *reeler* mice — *reelin* — was cloned in 1995¹. Reelin is an extracellular-matrix glycoprotein that is secreted by a few embryonic brain neurons, such as Cajal–Retzius cells in the marginal zone of the cerebral cortex². Yet most of the neurons that show the *reeler* phenotype do not express the *reelin* messenger RNA and protein^{3,4}, suggesting that Reelin acts at a distance in a juxtacrine fashion on target cells, radial glial cells and/or neurons.

The latest breakthrough is reported by Sheldon *et al.*⁵ and Howell *et al.*⁶ on pages 730 and 733 of this issue, and by Ware *et al.*⁷ in *Neuron*. The authors clearly identify the mouse *disabled homologue 1* (*mdab1*) gene as an essential component of the Reelin response in target neuronal cells. The story is a textbook example of convergent science, and it vividly illustrates the lightning speed of current molecular genetics.

The *scrambler* mutation was isolated a few years ago at the Jackson laboratory⁸. It produces a *reeler* phenotype but, whereas *reelin* maps on chromosome 5, *scrambler* maps on chromosome 4. *Yotari* (which is Japanese for tottering) is a similar mutation

which maps to the same locus as *scrambler*⁹. The *scrambler/yotari* locus was considered to be a prime candidate for a Reelin receptor, and it was eagerly pursued using positional cloning. But earlier this year, Howell and colleagues¹⁰ reported that *mdab1* is expressed in the embryonic brain and, like *scrambler*, it maps to mouse chromosome 4. *mdab1* is one of two mouse homologues of the *Drosophila* gene *disabled* which, in turn, was isolated in a screen for mutations that modify the neurological Abelson null phenotype in flies¹¹.

Although the map location of *mdab1* did not suggest an immediate link with *scrambler*, Howell *et al.*⁶ have now elegantly shown that when they inactivate the *mdab1* gene using homologous recombination, the result is a *reeler*-like phenotype. This finding,

communicated before publication, proved directly useful to the positional-cloning initiatives of Sheldon *et al.*⁵ and Ware *et al.*⁷, who demonstrated that *scrambler* and *yotari* are mutations of *mdab1*.

mdab1 mRNA and protein are expressed in neurons in the cortical plate, and in cerebellar Purkinje cells^{5,6} — two main targets of the *reeler* mutation. Although it is expressed in neurons that respond to Reelin, mDab1 is not a Reelin receptor, but a cytoplasmic protein that is phosphorylated during neural development. mDab1 binds to the Src-homology (SH2) domains of non-receptor tyrosine kinases such as Src, Fyn and Abl, and it has a protein interaction/phosphotyrosine-binding domain, providing potential for another class of protein–protein interactions.

The work of Sheldon *et al.*, Howell *et al.* and Ware *et al.* indicates that extracellular Reelin signals to nearby neurons by activating a kinase cascade. The involvement of kinases was already suspected through observations that mice deficient in cyclin-dependent

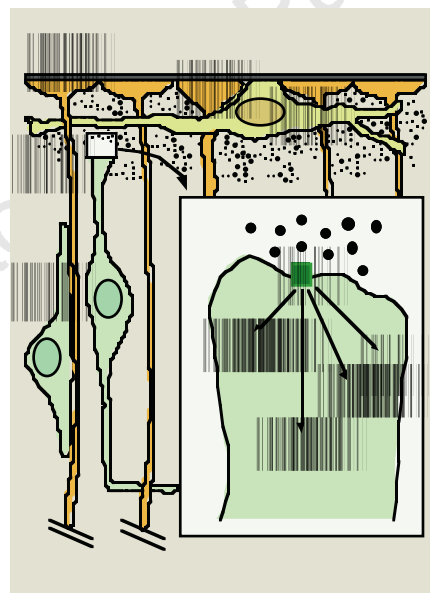


Figure 1 Model of Reelin action on developing cortex, based on the findings of Sheldon *et al.*⁵, Howell *et al.*⁶ and Ware *et al.*⁷. Immature neurons (light green) migrate along radial fibres (RF, orange). Reelin (dots) is secreted by Cajal–Retzius cells (CR, yellow) and incorporated in the local extracellular matrix. End-migration cortical-plate neurons (CP, dark green) respond to the presence of Reelin (which is sensed by their apical dendritic tip) by stopping migration, detaching from the RF and assuming a radial organization. The inset shows a neuronal tip. The mechanism by which this senses the Reelin signal is not known. Signal transduction and the neuronal response do, however, clearly require mDab1, Cdk5 in complex with its activator p35 (but also in association with another adaptor) and, presumably, hitherto unidentified molecules. PIA, meningeal surface and basement membrane; MZ, marginal zone.

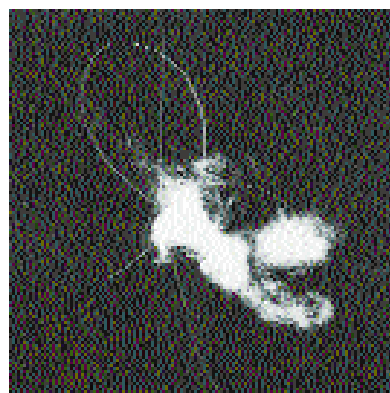
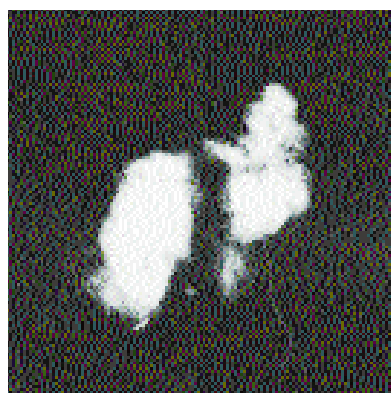
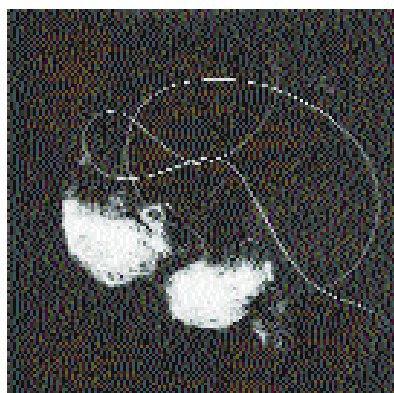
Another matter

Navel fluff

1: sailor (while at sea)

2: farmer

3: architect



kinase 5 (Cdk5) have a *reeler*-like phenotype¹² — Cdk5 is a serine/threonine kinase that is specifically expressed in postmitotic neurons. Moreover, cortical development does not proceed normally in mice that are deficient in p35, a Cdk5 activator¹³. The neurological anomalies in p35-knockout animals are more subtle than in *cdk5*-knockout mice, suggesting that proteins other than p35 help in the regulation of Cdk5.

Based on these latest observations, it is tempting to propose an updated model of Reelin action on migrating neurons in the developing cerebral cortex (Fig. 1). This model raises several questions. First, what are the transcriptional mechanisms that regulate Reelin expression? Certainly, they must be very accurate if we compare the high expression in Cajal–Retzius cells with the absence of expression in adjacent cortical-plate neurons. Second, how does Reelin act on target neurons? Is there a surface Reelin receptor coupled to intracellular transduction, or does Reelin interact less directly with other receptor systems? Third, how is the signal that is generated by Reelin translated into a cell response? A proper arrangement of the cytoskeleton is needed for the formation of cell patterns, so how does the presence or absence of Reelin affect this cellular organi-

zation? Interestingly, whereas Reelin is purely extracellular, the characterization of mDab1 provides a tool to attack some of these questions from the inside of the cell.

Given the pace of recent progress, it cannot be long before these questions are answered. However, our past experience with this thorny problem has taught us that answers are likely to be quite different from what we imagine. □

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Genomic imprinting

Making sense or antisense?

Wolf Reik and Miguel Constanca

Genomic imprinting is a genetic mechanism by which genes are expressed from the maternal or paternal chromosomes¹, and loss of imprinting is involved in a variety of diseases and cancers. All the imprinted genes that have been studied so far have regions in which the maternal and paternal DNA copies are methylated differently at CpG residues — cytosine–guanine base pairs. So DNA methylation is suspected to be the signal from the parental germ cells that results in the allele-specific expression of imprinted genes. Indeed, in mice that do not have methylation (because of mutation in the *methytransferase* gene), imprinting is altered².

The exciting work reported by Wutz *et al.*³ on page 745 of this issue now shows that differentially methylated regions in a specific imprinted gene (*Igf2r*; the maternally expressed gene that encodes the type-2 receptor for insulin-like growth factor) can carry a crucial imprinting signal. When this region is deleted from *Igf2r*, the gene loses its imprinting and is expressed regardless of parental origin. Moreover, the authors show that the deleted region (which is in an intron of *Igf2r*) is normally the promoter of an antisense transcript that is expressed from the paternal chromosome. Intriguingly, when the antisense gene is expressed, the sense is

not, and vice versa, indicating that some form of 'expression competition' regulates imprinting of *Igf2r*, as it apparently also does with other imprinted loci^{4,5}.

Methylation differences are found in two regions of the *Igf2r* gene. Region 1 (Fig. 1a, overleaf) is in the promoter, and it is methylated when the gene is not expressed. Region 2 is downstream in the second intron, and it is methylated in the expressed maternal copy. The maternal methylation patch (region 2) seems to originate in the germ cells, where eggs are methylated and sperm is not, leading to the suggestion that this region contains an 'imprinting box'. Moreover, because only the expressed allele is methylated, region 2 is thought to contain silencer sequences that can be suppressed by DNA methylation. Other imprinted genes have similar methylated regions in the expressed allele, indicating of similar mechanisms¹.

To show that the *Igf2r* gene contains local signals that are sufficient for imprinting, Wutz *et al.*³ introduced a (marked) copy of the gene on a yeast artificial chromosome (YAC) into transgenic mice. In three of the four transgenic lines studied, they observed proper imprinting, with expression on maternal transmission and repression on paternal transmission. Hence, the new study,



100 YEARS AGO

The latest number of the *American Naturalist* is the first which has appeared under the new editors. Dr Robert P. Bigelow, of Boston, is now the responsible editor "Every scientific man, as such" [he writes], "may well read two general scientific journals — the weekly scientific newspaper and the monthly review of scientific progress." The *American Naturalist* will aim at providing investigators with the latter form of scientific information. Authors of papers intended for beginners, such as "Some Birds of the Garden," "Some Common Weeds," are politely informed that their contributions are not wanted, and very technical works of interest to only a limited number of specialists will be declined. What the editors desire is scientific papers written by scientific people and of interest to scientific workers in more than one field. The desire is a praiseworthy one, and we hope the fulfilment of it will exceed the editors' expectations.

From *Nature* 14 October 1897.

50 YEARS AGO

The lecture on "The Organisation of Industrial Research" which Dr. R. P. Russell, president of the Standard Oil Development Co., delivered on June 9 to the Industrial Research Committee of the Federation of British Industries was... an outstanding contribution to what may be termed the philosophy of research. Research activity in his own Company, he said, began in 1919 with a group of twenty-six people: to-day the staff includes 2,456 technologists, engineers, assistants and clerical personnel all engaged exclusively on research and development projects, as well as several hundred working on laboratory phases of direct operating problems and an engineering staff of more than five hundred. Dr Russell computed that this expansion had brought a return of £15,400 of additional profit for each £1,000 expended on research and development.

From *Nature* 18 October 1947.

Many more abstracts like these can be found in *A Bedside Nature: Genius and Eccentricity in Science, 1869–1953*, a 266-page book edited by Walter Gratzer. Contact David Plant. e-mail: subscriptions@nature.com

along with work by Ainscough *et al.*⁴ — who use a similar YAC approach to study *Igf2-H19* imprinting — clearly shows that 'local' imprinting controls exist and are sufficient for proper imprinting. However, although the imprinting seen with the YACs is good it is perhaps not perfect (there are effects of position³ and copy number⁴), suggesting that regional control may also

be required to achieve perfect imprinting. Indeed, regional control mechanisms for imprinted gene clusters are inferred from 'imprinting mutations', which can disrupt imprinting in the cluster. These mutations, in 'imprinting centres', seem to underlie some of the imprinting diseases in humans (such as Beckwith–Wiedemann syndrome and Prader–Willi/Angelman syndromes)¹.

What are the local control elements that are needed for imprinting of the *Igf2r* YAC? When Wutz *et al.* deleted the maternally methylated region 2, *Igf2r* was expressed regardless of parental origin (Fig. 1b). So this region might, indeed, contain a silencer. Or does it? The authors also describe an antisense transcript that originates in region 2 and is expressed from the paternal chromosome. If you turn Fig. 1a upside-down, the antisense gene looks as if it has a normal promoter (sitting in a CpG island) which is silenced by maternal methylation, beginning in the egg. Antisense expression (from the non-methylated paternal chromosome) would presumably begin just after implantation of the embryo, when the switch from biallelic to monoallelic expression of *Igf2r* occurs.

If the antisense gene is involved in the switch from biallelic to monoallelic *Igf2r* expression, how does its expression lead to repression of *Igf2r* (and vice versa)? Perhaps the antisense transcript leads to some form of occlusion at the *Igf2r* promoter (Fig. 2a). Or maybe the antisense RNA locally inactivates the chromosome (Fig. 2b), by coating the chromatin in the same way as the RNA product of the *Xist* gene on the X chromosome^{6,7}. Finally, the promoters for the antisense transcript and *Igf2r* might share transcription factors that are in limiting supply or, as for *Igf2-H19*, they might compete for shared enhancers in *cis*^{4,5} (Fig. 2c). But although this simple flip-flop model between sense and antisense is appealing, it needs to be tested functionally. Also, *Igf2* and *H19* seem to be using a flip-flop system^{4,5}, but exceptions have been found. Such exceptions — which indicate additional levels of control — may also be expected in the *Igf2r* system.

There seem to be parallels between the *Igf2r* and *Igf2-H19* systems. But although a long antisense RNA could creep all the way (70 kilobase pairs) from *H19* to *Igf2*, in this system it is more likely that the two genes compete for shared enhancers. The 'local' imprinting control element has not yet been identified in the YAC transgenes⁴, but it has been pinpointed to upstream of the *H19* promoter⁸. However, the *Igf2* gene is also overlapped by an imprinted antisense transcript, the function of which is untested⁹.

How is DNA methylation in 'imprinting boxes' initiated and maintained? Wutz *et al.*³ provide a tantalizing glimpse of what might be happening. They made a mutation in the promoter of *Igf2r* which, on maternal transmission, leads to demethylation of region 2. As a result, the antisense gene is expressed from the maternal allele. This suggests that the *Igf2r* transcript is required to initiate and/or maintain the maternal methylation in region 2. So protection from demethylation — which seems to be the default state for CpG islands — is apparently afforded by

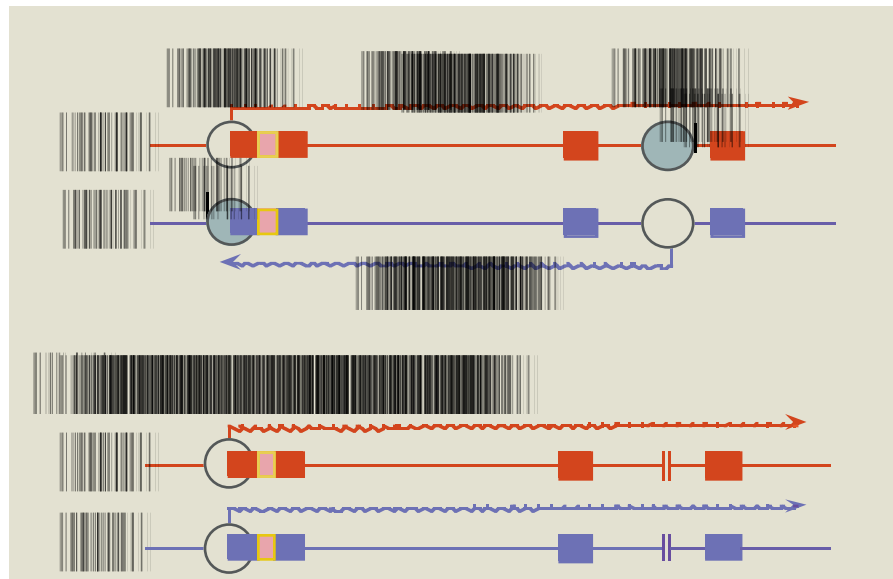


Figure 1 Wutz *et al.*³ have shown that differentially methylated regions in the *Igf2r* gene can carry a critical imprinting signal. They introduced a yeast artificial chromosome (YAC) transgene containing the whole *Igf2r* gene (filled boxes are exons) into mice. The coding region was modified (open box in the first exon) so that no IGF2R peptide was produced. Regions 1 and 2 are sites of differential methylation (grey; CH₃) — methylation of region 2 is introduced in the egg, whereas methylation of region 1 arises during embryonic development. a, Imprinting is maintained with the YAC transgene, with maternal (MAT) expression of the *Igf2r* sense transcript and paternal (PAT) expression of the antisense gene. b, Deletion of region 2 abolishes the expression of the antisense message and, hence, paternal repression.

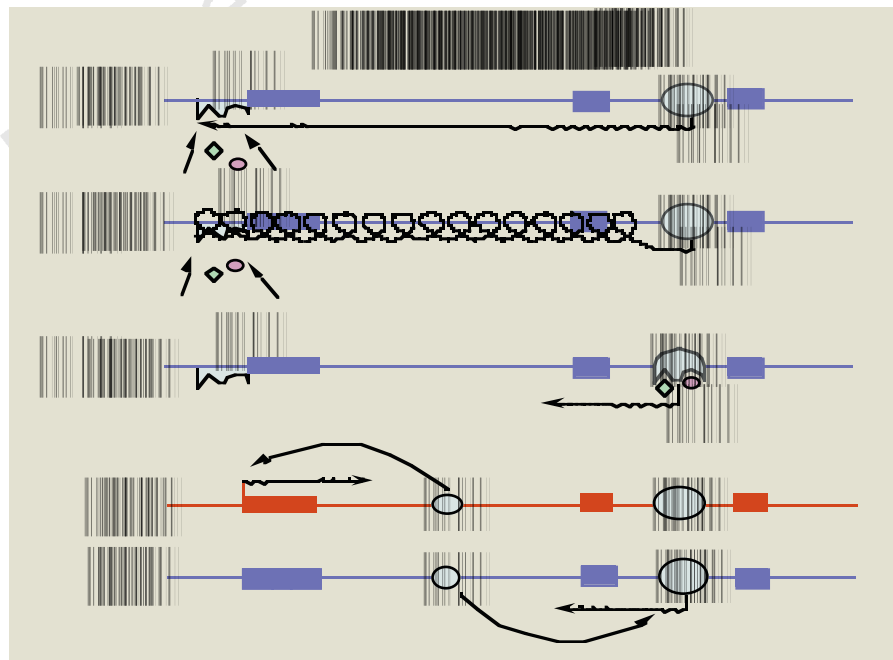


Figure 2 Models for paternal silencing of *Igf2r*. a, The antisense transcript occludes the *Igf2r* promoter (P), so transcription complexes cannot bind. b, The antisense transcript coats the paternal chromosome and leads to heterochromatinization. c, Competition for transcription factors or enhancers leads to repression of *Igf2r* when the antisense promoter is engaged.

Igf2r transcription through the methylated island.

But loss of maternal methylation in region 2 could also be explained by the transcription-factor competition model. If the promoter is mutated, transcription complexes cannot bind there. So, more transcription complexes may bind to the antisense promoter, leading to demethylation and, subsequently, expression of the antisense transcript.

It is still not clear what attracts egg-specific methylation to region 2 (or any other 'imprinting boxes'). Can the short transgenes containing region 2, which Wutz *et al.* show cannot maintain methylation, attract it in the first place? If so, are the direct-repeat sequences that are found near 'imprinting boxes' necessary to attract germline-specific methylation, which apparently uses different machinery to embryonic *de novo* methylation?

Finally, the human *IGF2R* gene also has a differentially methylated region 2, but is monoallelically expressed in few people and/or only at early fetal stages¹⁰. Hence, it could be predicted that the antisense tran-

script exists in some human *IGF2R* alleles but not others, that its structure is polymorphic or, perhaps, that it has a different spatio-temporal regulation to the one that exists in the mouse. One thing is clear — imprinting has brought (and will continue to provide us with) interesting and surprising insights into the complex mechanisms of epigenetic gene regulation. □

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Particle physics

Panning for gold at the K stream

Frank Wilczek

In 1947, while studying cloud-chamber pictures of cosmic-ray showers, Rochester and Butler¹ found two pictures, among 50 exposures, that seemed to represent the decay of two unstable particles. The tracks were quite different from anything seen before, and were not anticipated by any theory. The researchers assumed that the pictures showed some kind of meson decay; they continued to make observations for two more years, but without further success.

This summer, almost exactly 50 years after those humble beginnings, a new milestone result in the study of K-meson decays was announced — an achievement that took several years of honing technique and gathering data, and which would have astonished Rochester and Butler. In collaboration with colleagues elsewhere in the United States and in Japan and Canada, scientists at Brookhaven National Laboratory have observed a decay mode of the positive K meson that occurs approximately once in ten billion decays; the details have just been published in *Physical Review Letters*². Specifically, the team concerned have for the first time identified an occurrence of $K^+ \rightarrow \pi^+ \nu \bar{\nu}$, the decay of a positively charged K into a positively charged pi meson, a neutrino and an antineutrino.

So what does it mean? Physics has been stuck with its remarkable success in producing a Standard Model of the interactions between the different fundamental particles; for over two decades, this theory has gone

from triumph to triumph, with no sign of any discrepancy with experiment (neutrino oscillations, if confirmed, would require a significant but only small extension of the Standard Model). This situation has become frustrating both to theorists, who would, for instance, like to see concrete evidence for the relevance of their exciting ideas about unification and supersymmetry, and of course to experimentalists, who grow weary of verifying old theories. A good way to look for something new, one that is complementary to the traditional push towards ever higher energies, is to look for rare processes at low energy. This approach provides a test of our theories, and a handle on new processes at high energies or short distances, that in the past has proved extremely informative.

There are quite definite rules for calculating the probability (or quantum-mechanical amplitude) of a process depicted in a Feynman diagram (Fig. 1), so the pictures exist at two levels: they are both portraits and recipes. Here the K^+ meson, made up of u (up) quark and s (strange) antiquark, fluctuates through a loop including heavy 'virtual' particles before materializing into a π (u quark and d (down) antiquark) and neutrinos. A W boson is necessarily involved, to change one type of quark into another, as is a Z boson, to couple to the neutrinos. Because the real versions of these particles are heavy, their virtual analogues arise only rarely as quantum fluctuations in the vacuum. That

is why the decay shown is very rare.

The orange particle, shown with a question mark in Fig. 1, can be either a u or a c (charmed) quark. Before 1970, it was considered that only the u quark would contribute, and the predicted value for this and related processes was much too large to be compatible with experiment. Then Glashow, Iliopoulos and Maiani³ proposed that an additional contribution from the c quark cancelled out most of the u quark's contribution. This suggestion was brilliantly confirmed by the experimental discovery of real c quarks in 1974. There is also a similar contribution with t (top) quarks, which (after u and c have mostly cancelled) actually dominates the predicted rate.

Looking at Fig. 1, one is struck by how many different, fundamental components of the Standard Model come into play in making this K-meson decay occur. Indeed, if we include gluons, shown as the squiggles in Fig. 1, to hold the quarks in the K and pi mesons together, they all do. It is quite amazing that this intricate description really works.

The big question, to be answered by experiments that are underway, is whether additional, unknown particles also contribute significantly. Just as the existence of Neptune, a planet beyond the then standard model of the Solar System, was inferred from its small but noticeable influence on the orbit of Uranus, the existence of heavy particles and phenomena beyond the Standard Model of fundamental physics might first be revealed by tiny effects on the behaviour of the known particles.

With just one event observed so far, however, no very definite conclusion can be drawn. The Standard Model predicts⁴ that

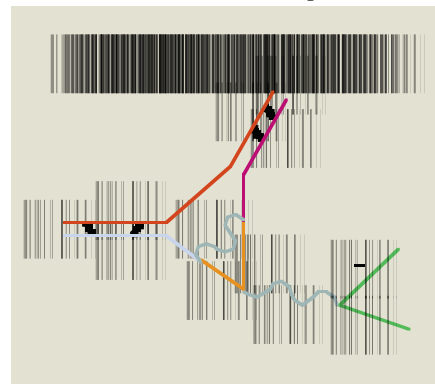


Figure 1 Virtual particles and the very rare case of K^+ -meson decay. In the language of quantum field theory, the decay of a particle is described as a series of events depicted in a Feynman diagram. This is the diagram describing a type of K-meson decay, resulting in a pi meson and neutrinos, which has just been observed experimentally for the first time². ν , neutrino; $\bar{\nu}$, antineutrino; Z and W, bosons; s, u, d, strange, up and down quarks. The particle shown in orange, with a query, can be a u, charmed or top quark. The squiggles holding the quarks in the K and pi meson together represent gluons.

$K^+ \rightarrow \pi^+ \nu \bar{\nu}$ occurs with a branching ratio $1.0 \pm 0.1 \times 10^{-10}$, whereas the one reported event corresponds to $4.2^{+9.7}_{-3.5} \times 10^{-10}$. If there is no physics beyond the Standard Model at work, the experimenters have been a little lucky to see an event so soon. And, of course, if the event is the first tangible hint of physics beyond the Standard Model, for example in reflecting the influence of supersymmetric particles, they will have been luckier still. Only half of the existing data have been analysed so far; the rest will be scrutinized over the next few months.

Beyond that, it will be essential to gather much more data, so that a decisive confrontation with the expectations of the Standard Model becomes possible. That confrontation is eagerly awaited. □

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G proteins

The arginine finger strikes again

Henry R. Bourne

The Ras protein and members of its family are G (guanine-nucleotide-binding) proteins, or GTPase enzymes, that play pivotal roles in transmitting regulatory signals between the cell surface and the nucleus. Ras itself, for instance, is involved in control of cell proliferation and differentiation. These proteins are turned on by binding GTP, and off when the GTP is hydrolysed to GDP by GTPase-activating proteins, or GAPs. But how does this crucial turn-off mechanism work?

Now we know, thanks to reports^{1–3} of the three-dimensional structures of Ras and two other GTPases, crystallized in the grips of different GAPs. The crystals reveal elegantly crafted examples of convergent evolution — three structurally distinct protein machines that use identical mechanisms to promote GTP hydrolysis. Details of the structures also point to hitherto unknown regulatory functions for GAP-related switches, and they show, at atomic resolution, why Ras mutations cause 25 per cent of human cancers.

The structures, respectively, are of the α -subunit, $G_{\alpha_{11}}$, of a trimeric G protein in combination with RGS4 (a regulator of G-protein-signalling protein)¹; H-Ras with a catalytic fragment of RasGAP²; and, on page 758 of this issue³, RhoGAP in a complex with RhoA, a GTPase that controls remodelling of the actin cytoskeleton.

Efficiency of an enzyme reaction depends on the enzyme's ability to stabilize substrate atoms in a very specific arrangement, the transition state, which allows the reaction to proceed. The transition state's high energy makes it too unstable to be captured in a crystal structure. Instead, investigators crystallized GTPase–GAP complexes in a solution containing GDP and a combination of ions (Al^{3+} and F^-), which form a planar complex thought to mimic the atomic arrangement of GTP's γ -phosphate in the transition state (see box, overleaf).

The three GTP-hydrolysing machines form nearly identical webs of hydrogen

bonds that hold key substrate atoms in the configuration required for GTP hydrolysis (see box). In each complex, one protein (Ras, RhoA or $G_{\alpha_{11}}$) shares with its counterparts in the other machines a common GTP-binding fold and stabilizes the transition state with the same set of identically positioned amino acids — with a single exception, an arginine residue whose guanidinium group interacts with the same substrate atoms in each transition state. Like fingers of different hands pointing at the same object, the arginines stick into the active site from different directions (see box).

The arginine fingers belong to three structurally different hands — RasGAP, RhoGAP and a built-in domain of $G_{\alpha_{11}}$. In this last case, as in all G α proteins, the finger projects from a loop that connects the built-in extra domain to the GTP-binding fold. All three hands grasp GTPases that evolved from a common GTP-binding precursor and share the same 3D architecture. In contrast, convergent evolution reshaped three α -helical but otherwise unrelated protein folds to produce morphologically different hands able to point an arginine finger with equal precision. Despite their differing evolutionary origin, arginines pointed by RasGAP (see box; blue) and RhoGAP (cyan) occupy similar positions in the active sites of their respective targets. The built-in domain of $G_{\alpha_{11}}$ points its finger (green) from almost the opposite direction; nonetheless, the position of its key guanidinium group is almost identical to those of RasGAP and RhoGAP.

In all three cases, amputation of the arginine finger creates a machine that hydrolyses GTP and turns off transmitted signals very slowly, as shown by the effects of missense mutations in the appropriate arginine codons of RasGAP⁴, RhoGAP⁵ and G_{α} proteins^{6,7}. Indeed, such mutations in G_{α_s} , a G α structurally similar to $G_{\alpha_{11}}$, cause endocrine tumours⁷ by triggering excessive synthesis of a second messenger, cyclic AMP.

When RasGAP is not available to deliver

its arginine finger to the active site in *trans*, Ras by itself hydrolyses only one GTP every 35 minutes. In contrast, the built-in arginine finger of G_{α} proteins allows them to hydrolyse GTP more rapidly (at about 2–4 min^{−1})^{7,8}. In the presence of the appropriate partner — the right GAP or an RGS protein — Ras, RhoA and $G_{\alpha_{11}}$ all hydrolyse GTP much faster, at between 10² and 10³ min^{−1}.

This remarkable acceleration attests to a second functionally essential feature of GTP-hydrolysing machines, also created by convergent evolution: enhanced stability of other amino acids (not just the arginine finger) at the active site. The additional amino acids are located in two regions of the GTP-binding fold, switch 1 and switch 2. Their stability is critical: to steady the GTPase transition state, these residues must hold an attacking nucleophilic water molecule and the γ - and β -phosphates of GTP in precisely the right relation to one another. This stability is greatly increased in proteins gripped by RGS4 or the appropriate GAP, as shown by better-defined electron densities of switch 1 and switch 2 residues in crystals containing the partner proteins^{1–3}.

The stabilizing embraces of GAPs and RGS for their GTPase partners differ in detail, because the GAPs and RGS evolved from structurally different precursors. For instance, the side chain (see box; magenta) of a conserved glutamine residue in switch 2 must be in exactly the right place to position the attacking water molecule, or GTP will not be hydrolysed. Both RasGAP and RhoGAP use the 'knuckle' (main-chain carbonyl) of the arginine finger to stabilize the side chain of this glutamine^{2,3}. Because the knuckle is out of reach in $G_{\alpha_{11}}$, RGS4 accomplishes the same end with a different amino acid¹.

Convergent structural evolution produces many opportunities to exploit these off switches in cell regulation. As we have seen, evolution engineered into trimeric G proteins an ability to switch signals off at two different rates. This was accomplished by supplying the arginine finger from a built-in G_{α} domain and by assigning the other GAP function, stabilization of amino acids in the active site, to a separate hand — that is, an RGS protein (or in some cases to a downstream effector, as exemplified by phospholipase-C β ⁹, which turns off its regulator, G_{α_q} -GTP). For the G_i -regulated K^+ channels that control brain synapses and heart rate, the accelerated turn-off rate provided by RGS proteins is physiologically essential. When a slower tempo would suffice, RGS proteins are presumably unnecessary — for instance, to shut off G_{α_s} -mediated signals that promote conversion of liver glycogen into glucose.

More subtle variations may also furnish opportunities for regulation. Binding of

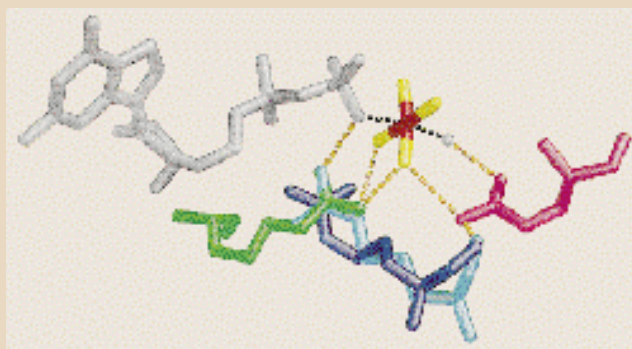
How converging fingers keep GTP in line

The figure here shows a simplified view of how arginine fingers stabilize the GTPase-reaction transition state. Depicted are the GDP (grey), AlF_4^- (a red aluminium surrounded by four yellow fluorine atoms), the attacking water (grey) and the conserved glutamine residue (magenta) as seen in the $\text{GDP}:\text{AlF}_4^-$ -bound structure of the $\text{G}\alpha_{11}$ -RGS4 complex¹.

In the three distinct GTP-hydrolysing machines¹⁻³ discussed in the main text, arginine fingers converge on the same point, stabilizing γ -phosphate oxygens and the leaving group. The finger pointed in *cis* by $\text{G}\alpha_{11}$ (green) approaches from one direction, while arginines supplied by RasGAP (blue) and RhoGAP (cyan) point from the opposite direction.

AlF_3 , which was found in the Ras-RasGAP structure², presumably mimics the transition state more accurately than does the AlF_4^- shown here, which was seen in the $\text{G}\alpha_{11}$ -RGS4 and RhoA-RhoGAP crystals¹³.

The transition state is bipyramidal, with a central phosphorus (the Al^{3+} ion in crystals) located equidistant from the leaving group (an oxygen of GDP's β -phosphate) and an attacking water molecule, which is destined to donate an oxygen to form a product of the GTPase reaction, inorganic phosphate. A straight line (black dots), perpendicular to the plane formed by the phosphorus and its three oxygens, connects the leaving group, phosphorus and water. The position of every atom in the



transition state is stabilized by a network of hydrogen bonds connecting it to a Mg^{2+} ion and/or neighbouring amino acids from the GTP-binding protein. This network is omitted here, except for bonds involving the arginine of $\text{G}\alpha_{11}$ and bonds connecting the glutamine to the water and AlF_4^- . In addition, arginine 'knuckles' (main-chain carbonyls)

positioned by the GAPs stabilize the amide group of the glutamine's side chain; this stabilizing influence is represented by an imaginary bond connecting the arginine main chain of RhoGAP to the glutamine of $\text{G}\alpha_{11}$. (Coordinates of crystal structures kindly provided by S. Sprang¹, A. Wittinghofer² and S. J. Smerdon³. Elaine Meng helped in making the graphic.)

H.R.B.

GDP, Al^{3+} and F^- promotes configurations of switch 1 and switch 2 different from those seen in the 'ground state' of Ras, Rho or $\text{G}\alpha_{11}$ bound to hydrolysis-resistant GTP analogues¹⁻³. Both configurations differ from the GDP-bound inactive conformation. Only one can support the transition state of the GTPase reaction, however, while either may increase or decrease the signalling protein's affinity for effectors and other proteins.

Not surprisingly, other proteins can sometimes tell the difference between the two GTP-bound conformations. For instance, RhoGAP interacts differently with Rho proteins depending on whether the nucleotide-binding site contains $\text{GDP}:\text{AlF}_4^-$ or a GTP analogue. The GAP binds to the same switch regions in both complexes, but in the latter (as reported⁵ in crystals of RhoGAP bound to Cdc42p, a close relative of RhoA) it fails to stabilize residues in the active site, is orientated at a different angle with respect to its partner, and points the arginine finger in an inappropriate direction for stabilizing the transition state. Crystal structures and biochemical evidence indicate that the transition and ground states differ in Ras and $\text{G}\alpha$ also^{1,2,4}.

We can imagine several uses for the ability of RGS and GAP proteins to discriminate between the ground and transition states. Other signalling pathways could increase (for instance, by phosphorylation) the relative binding affinity of an RGS or a GAP for its partner's ground state, in order to pro-

duce a complex in which the GTP-binding component is sequestered but remains in the GTP-bound ground state. Sequestration of the signal transmitter would be expected to damp signal transmission, but might paradoxically enhance it — at least in the case of signals carried by the $\beta\gamma$ subunit of G_i , because such signals normally terminate when $\beta\gamma$ reassociates with $\text{G}\alpha_i\text{GDP}$ after GTP has been hydrolysed.

The quite different orientations of RhoGAP with respect to its partner in the ground and transition states suggest that a GTP-hydrolysing machine might monitor the relative positions of its partners in space with considerable precision. Hydrolysis of GTP by elongation factor Tu (EF-Tu) depends on interaction of the protein with the mRNA-programmed ribosome^{10,11}. Perhaps a tight anticodon-codon interaction between the amino-acyl tRNA bound to EF-Tu and the mRNA positions the ribosome's arginine finger to trigger hydrolysis of GTP bound to the elongation factor.

Finally, the Ras-RasGAP structure shows why mutations that replace glycine-12 in Ras cause cancer: Ras-mediated mitogenic signals cannot be turned off, because the side chain of any other amino acid at position 12 would displace RasGAP's arginine finger and prevent proper tickling of the γ -phosphate². The affinity of RasGAP for the mutant Ras protein is unchanged¹², however. This suggests that the embrace of RasGAP may be able to stabilize configurations of active-site residues in switch 1 and switch 2 even

when its arginine finger can't push the right button.

If so, might it be possible to accelerate GTP hydrolysis by inserting a surrogate finger into the GTPase active site, in the form of a drug? Although several molecules containing amidino groups fail to restore the ability of 'fingerless' RasGAP to stimulate GTP hydrolysis by wild-type Ras *in vitro* (A. Wittinghofer, personal communication), measuring GTPase activity of a glycine-12 Ras mutant in the presence of wild-type RasGAP might be used to identify the right compound in a chemical library. Alternatively, persuading a complex of mutant Ras and RasGAP to form properly diffracting crystals could guide design of an effective prosthetic finger. □

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Ceramics

Tough cookery

Derek Thompson

Materials scientists are used to identifying a material from the shape and form of the grains in its microstructure — there is even a tendency to assume that a particular phase always occurs in the same crystal form (needles, plates, spheres ...). On page 701 of this issue¹, Chen and Rosenflanz show how this assumption is wrong in one particularly interesting case. Whereas the ceramic α -SiAlON usually occurs as equiaxial grains, it can form as needles under different conditions of nucleation and growth. This achievement is of immediate practical relevance because α -SiAlON is extremely hard — even harder than β -SiAlON, which is used to make such things as turbochargers and other engine components. But until now α -SiAlON ceramics have always been brittle, because of their equiaxial microstructure.

The search for tough ceramics is unending. Usually, if a new toughening mechanism is identified, it will only work over a limited range of temperature, or it will be at the expense of strength or hardness (see box for the distinctions between these three quantities). Silicon nitride and its related SiAlON ceramics² have an advantage over competing materials because, as well as having high strength (up to 1,000 MPa) and hardness (15–20 GPa), they can form microstructures that also have slightly better toughness (in the range 6–9 MPa m^{1/2}); moreover, these properties are retained up to temperatures beyond 1,000 °C.

SiAlON ceramics were discovered some 25 years ago³, and are now established materials — used in cutting tools, wire drawing, dies, tappets and shot blast nozzles, for example. As with silicon nitride, there are two forms, α and β , with atomic arrangements related to the equivalent α and β forms of silicon nitride (differing only in the stacking sequence of successive atomic planes). The most common commercial form is β -SiAlON, in which a certain proportion of the silicon atoms in β -silicon nitride have been replaced by aluminium, valency balance

being retained by replacement of the same number of nitrogen atoms by oxygen.

In a similar way, α -SiAlON is related to α -silicon nitride⁴, with silicon replaced by aluminium. Even though some nitrogen is replaced by oxygen, this is less than is needed to balance valencies, and the final valency balance is achieved by incorporating more cations (Li, Mg, Ca, Y and most rare earths) into large interstices in the structure. α -SiAlONs are normally produced by sintering — baking powdered mixtures of alumina, aluminium nitride and a metal oxide with conventional 95% α -, 5% β -silicon-nitride powder — and the product forms as small equiaxial α -SiAlON grains. The hardness of α -based materials is always larger than β -based, for both silicon nitride and SiAlONs.

But β -SiAlON is tougher, because its final microstructure contains some elongated grains of β -Si₃N₄ (Fig. 1). These form because the starting silicon-nitride powder always contains ~5% of β -silicon nitride, in the form of tiny needles. At the temperatures used to fire the ceramic, the α -silicon nitride is unstable and transforms to the β -form, mostly as small irregular grains — but some precipitates onto the original β needles, which thus grow into much larger needles, until adjacent large needles meet. These increase toughness in exactly the same way as carbon fibres reinforce composite ceramics.

Chen and Rosenflanz¹ have now shown that α silicon nitride and α -SiAlON ceramics can be made with a similar tough whiskery microstructure. They mix a silicon-nitride powder consisting of 95% β -Si₃N₄ and 5% α -Si₃N₄ with alumina, aluminium-nitride and rare-earth-oxide powders. Because of the different starting mix the β -Si₃N₄ is now unstable and reacts with the other ingredients to form α -SiAlON, which again forms partly as small equiaxial grains and partly deposits itself on the existing α -silicon-nitride needles (Fig. 1a on page 702).

The authors produce an α -SiAlON mat-

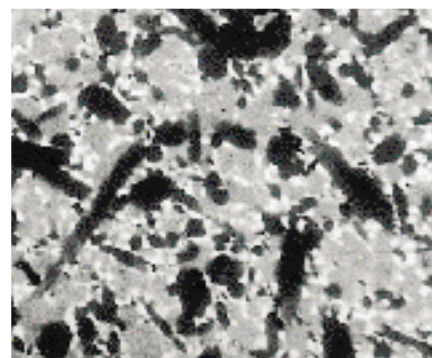


Figure 1 Typical microstructure of a mixed α - β SiAlON ceramic. Equiaxial α -SiAlON grains (grey) and elongated β -SiAlON (black) are interspersed with pockets of grain-boundary glass (white). The new ceramic is all α -SiAlON, much of which is elongated, giving the overall material an exceptional combination of toughness and hardness.

Table 1 Rival materials

	Hardness (GPa)	Strength (MPa)	Fracture toughness (MPa m ^{1/2})
α -SiAlON (conventional)	≈20	500–700	4–5
β -SiAlON	≈15	700–1,000	6–9
Alumina	≈15	300–400	3–4
Silicon carbide	≈25	400–500	2–4
Glass	≈10	Variable	≈1
Mild steel	1.5	200	>100

erial with very similar strength and toughness to previous β -silicon-nitride materials, and with the increased hardness of the α -form. Because nitrogen ceramics find application as parts that need to be wear-resistant, it is important to have a good combination of strength, hardness and toughness, and the new materials offer the best combination of all these three properties so far. Performance is retained up to temperatures of at least 1,000 °C, comfortably above working temperatures for most wear applications.

Moreover, the shape of the grains can be controlled by the choice of sintering additive. Rare earths are often used for stabilizing α -SiAlONs, and Chen and Rosenflanz have shown that the ease of formation of elongated α needles varies with rare-earth cation (as the wetting properties of Ln-Si-Al-O-N liquids vary⁵ with rare-earth cation Ln). It is easy to produce needle-shaped α grains in α -SiAlONs containing low-atomic-number rare earths, but more difficult with higher atomic numbers. Chen and Rosenflanz show that this is a nucleation problem, and that by careful control of the firing sequence it is also possible to produce elongated α -SiAlON grains using the higher-atomic-number rare earths.

This work reminds us that the morphology of crystals is not a universal constant, but is influenced by the local conditions during growth. Because the mechanical properties of materials are so strongly influenced by

Hard definitions

Strength, hardness and toughness are by no means synonymous. **Strength** is a measure of the ability of a material to resist applied stress (compression or tension). **Hardness** is the resistance to indentation, usually calculated from the size of indentation made by

a shaped diamond crystal pressed with a standard force into the surface of the material. **Toughness**, roughly speaking, is the amount of energy needed to break a piece of material, and is more complicated to quantify. For a brittle material it can be measured either

as the energy absorbed per unit area of crack, G , or (more commonly for engineering ceramics) as $(EG)^{1/2}$, where E is Young's modulus. This is called the 'critical stress intensity factor' or the 'fracture toughness', and has units of MPa m^{1/2}.

D.T.

their microstructure, it is surprising that more effort has not been put into looking at the effects that the grain shape and size, and the proportions of individual phases in the starting powders, have on the morphology of the finished product. The discovery of whiskery α -SiAlON opens up new possibilities for optimizing the strength, hardness and toughness of nitrogen ceramics—and at very little extra cost to the manufacturer. □

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Alzheimer's disease

The ins and outs of amyloid- β

Konrad Beyreuther and Colin L. Masters

The devastating consequences of Alzheimer's disease — marked by extracellular senile plaques with fibrils of amyloid- β peptide (A β) and intraneuronal tangles of polymerized tau protein — are experienced by more and more elderly people in the Western world. The latest step towards understanding the underlying causes is reported by Yan *et al.*¹ on page 689 of this issue. They have identified a possible mechanism to explain how A β could damage neurons, and they show that A β interacts with a hitherto unknown endoplasmic-reticulum-associated binding protein, ERAB.

We already have compelling evidence that A β may be at the root of neurodegeneration: mutations have been found in three genes — the amyloid precursor-protein (APP) gene on chromosome 21, and the genes for presenilin 1 (PS1) and presenilin 2 (PS2) on chromosomes 14 and 1, respectively — that segregate with rare forms of early-onset autosomal-dominant familial Alzheimer's disease. These mutations result in increased production² of the APP cleavage product A β 42, which is a main constituent of plaques but is not found in the paired helical filaments of tangles^{3–5}. Moreover, all of the mutations in the APP gene are within the A β region. So although A β seems to be implicated in neuropathogenesis and neurodegeneration, the missing link revolves around how it interferes with neuronal function and tangle formation.

Using the yeast two-hybrid system, Yan *et al.*¹ isolated four positive clones (one from a human brain and three from a HeLa complementary DNA library) coding for an A β binding protein. All of the clones turned out to be derived from the ERAB gene on the human X chromosome. The 262-amino-acid ERAB protein shares a series of structural motifs with the family of short-chain alcohol dehydrogenases that includes hydroxysteroid dehydrogenases and acetoacetyl-CoA reductases. Because these enzymes are involved in cholesterol biosynthesis, the authors reasoned that by working out what ERAB does, they might find the connection between the ApoE predisposition genetics (one of three allotypes of ApoE carries a higher risk⁶),

cholesterol, A β and Alzheimer's disease.

ERAB localizes to the endoplasmic reticulum, despite the absence of a signal or transmembrane sequence. In the presence of A β , it is translocated to the inner aspect of the plasma membrane, and it no longer associates with the endoplasmic reticulum of cultured neurons. There is strong neuron ERAB reactivity in the brains of patients with Alzheimer's disease, yet it is almost absent in normal brain. Moreover, ERAB is found near β -amyloid plaques, and the cellular toxicity of A β can be reduced by blocking ERAB and increased by its overexpression. These factors led Yan *et al.* to propose that the intracellular interaction of ERAB with A β may contribute to neuronal dysfunction in Alzheimer's disease.

How does this discovery fit into the broad context of plaques, tangles and neurodegeneration? Ever since A β 42 was first found in them⁴, extracellular plaques have been thought to be sources of toxicity, causing local cell death and, on a brain-wide scale, the extensive cortical degeneration of Alzheimer's disease⁷. But there are now several reasons to doubt this simplistic account. First, in a diseased brain, pronounced cell death is not found in the immediate vicinity of the plaques — indeed, transgenic mice given extra copies of the APP gene produce large quantities of amyloid and develop plaques, but without concomitant neuronal loss⁸. Second, although genetic evidence supports the idea that overproduction of A β 42 may be the cause of Alzheimer's disease, levels of A β are not increased in body fluids of most sporadic or familial patients with late-onset disease². Third, so far, only neurons have been found to generate secretory A β and abundant levels of intracellular A β 42 (refs 9, 10), produced in the endoplasmic reticulum¹¹. Finally, the A β domain is required for axonal sorting — a somewhat surprising biological function for this part of APP¹².

An attractive hypothesis for the role of A β in intraneuronal sorting would be that APP interacts directly, through its A β domain, with a sorting receptor in order to be passaged into axonal transport vesicles. Interaction of A β with the sorting machinery could

inhibit this process by interfering with axonal transport of other proteins such as tau or α -synuclein, which are synthesized at somatodendritic ribosomes. This could explain why tangles, or Lewy bodies in Parkinson's disease, are formed in these compartments^{5,13}.

The interactions of intracellular A β with ERAB could regulate the relative utility of the APP sorting receptor, providing a link between neuronal sorting and dysfunction. But any interaction of ERAB with A β would require a membrane abnormality: whereas ERAB is bound to the cytoplasmic aspect of the endoplasmic reticulum, A β 42 is generated intracellularly (in neurons), within the lumen of the endoplasmic reticulum^{9,11}. Such an abnormality could be brought about by A β -induced oxidative stress or damage to membranes¹⁴. Or, it could be started by locally increased production of A β 42, owing to impaired transport of APP out of the endoplasmic reticulum, or prolonged residence of APP within it^{11,12,15}.

The work of Yan *et al.*¹ indicates that any A β 42 leaking out of the endoplasmic reticulum and binding to ERAB would lead to the translocation of ERAB–A β complexes to the plasma membrane (Fig. 1). Indeed, their study provides the first molecular evidence that could link intracellular and extracellular A β to neurodegeneration. As well as initiating neurodegeneration, this translocation could pave the way for extracellular A β to join in the process (Fig. 1). Deposition of extracellular A β in plaques would then provide an almost unlimited supply of interaction partners for ERAB, producing a chronic seques-

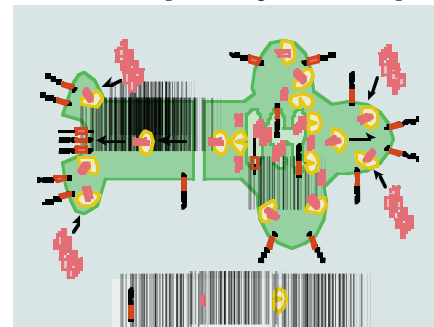


Figure 1 Yan *et al.*¹ have identified an endoplasmic-reticulum (ER)-associated binding protein (ERAB) which binds to amyloid- β peptide (A β). In the absence of cytosolic A β , ERAB seems to be associated with the cytoplasmic aspect of the endoplasmic reticulum. When A β is added to cultured neurons, ERAB rapidly translocates to the inner part of the plasma membrane. Where and how the ERAB–A β complexes become toxic is not clear but, in neurons (which express higher levels of ERAB than peripheral cells), toxicity of A β is prevented by liposome-mediated introduction of blocking anti-ERAB antibody fragments. This suggests that the induction of cell dysfunction by A β depends on the presence of ERAB–A β complexes. APP, amyloid precursor protein; A β 42, the product of cleavages within the ecto- and transmembrane domain of APP.

tration of ERAB from the endoplasmic reticulum to the plasma membrane. This may perturb the function of ERAB, which would be vital to the cell if it were linked to cholesterol biosynthesis, or if it activated inappropriate signal-transduction events leading to the induction of cell-death programmes.

The discovery of ERAB as an intraneuronal A β binding protein, and its possible involvement in Alzheimer's disease, provides a new tool to advance our understanding of the part played by A β in the convoluted pathways that lead to tangle formation, neuronal degeneration, dementia and death. Whether in or out of the neuron, A β means trouble. $\square$

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Geophysics

A magnetic reversal record

Ronald T. Merrill

The Earth's magnetic field has reversed polarity hundreds of times in the past, the most recent, well-documented event (the Brunhes/Matuyama reversal) occurring about 780,000 years ago. The duration of a reversal is generally thought to be between 1,000 and 6,000 years, during which time the average intensity of the field at Earth's surface is estimated to decrease to about a quarter of its usual value^{1,2}. What happens to the direction of the field during a reversal is controversial, and on page 712 of this issue³ Channell and Lehman set out to tackle the issue. They present some of the highest-resolution data ever obtained from marine-sediment records of two reversals — the Brunhes/Matuyama, and the one preceding it which occurred about 990,000 years ago.

Earth's main magnetic field is associated with electric currents in the iron-rich outer core, which occupies a depth range between 2,900 and 5,150 km. Nevertheless it is convenient, and for present purposes² is mathematically justifiable, to describe the field as originating from dipole and nondipole sources at Earth's centre. The dipole source can be pictured as a bar magnet, currently tilted 11° with respect to Earth's rotation axis, and accounts for about 80 per cent of the present field at Earth's surface. The rest essentially comes from the more complex nondipole sources. The geomagnetic poles are the two points on Earth's surface that lie along the axis of the dipole. The field is said to have normal polarity when the north geomagnetic pole lies above 50° N latitude, to have reverse polarity when it lies below 50° S, and to be transitional in between.

Past geomagnetic poles are estimated from the primary magnetization in rocks, which is usually acquired parallel to the palaeofield when a rock forms. One estimate

of the palaeofield's north geomagnetic pole (the virtual geomagnetic pole, or VGP) is obtained by assuming the field is perfectly dipolar. If this assumption were true, the VGPs obtained from rocks taken anywhere on Earth's surface would coincide, apart from experimental error. They would not coincide if the field were nondipolar. So we can get information on the character of the transitional field by examining the appropriate VGPs. This may appear to be straightforward, but the subject has a 30-year history of conflict. One area of disagreement concerns which rocks can be used to obtain the most reliable VGP estimates; a second involves the interpretations of the transitional VGPs.

Overall, the debate can be illustrated by three of the most popular 'models'. The first uses data from sedimentary rocks and holds that VGPs are confined to two antipodal longitudinal bands, each of which has a width of around 40° (refs 4, 5). According to this view, the VGPs for different reversals that occurred within the past ten million years occupy the same longitude bands. A difficulty, however, is that the primary magnetization in most sediments is acquired during deposition and compaction, and deposition often occurs at rates of about a centimetre every thousand years. So the magnetization in a particular sample is an average over a time that can be a significant fraction of the reversal transition time; that is, the sample is a filter that can lead to a substantial smoothing of the changes in the magnetic field. Moreover, sediments can be affected by problems that can confound interpretation, such as the presence of an undetected secondary magnetization, acquired after the rocks formed.

The second model uses data from lava flows, rocks that usually have fewer such problems. Advocates of this model conclude

that there are certain 'hang-up' points for the field during reversals that are manifested in a grouping of VGPs into a few geographical clusters^{6,7}. The same clusters, runs the argument, exist for different reversals. But the primary magnetization in lava flows is acquired during cooling, which usually occurs very quickly relative to the time of a reversal transition; so, unlike the record from sediments, that in lava flows is discontinuous. Some critics have also argued that there have often been bursts in igneous activity, followed by intervals of no activity, which may give rise to VGP clusters even when the actual magnetic field changed smoothly.

A third model emerged from an analysis⁸ of 362 transitional VGPs from 121 volcanic units that are less than 16 million years old. Here, the authors concluded that there is no persistent pattern for different reversals. Objections to this theory are that the time span used may be too long and that some of the transitional VGPs may not have occurred during a reversal.

Why would one wish to know which, if any, of these models is correct? Most obviously, there is natural curiosity as to what happens during a reversal transition. Beyond that, theories for the origin of Earth's magnetic field implicitly assume the third model. Some spatial bias in the boundary conditions for the outer core seems to be required if either of the first two models is correct²; if this is so, one then wants to know its origin, because the bias is likely to provide information on processes that have occurred, or are now occurring, just above the core. There is also the question of whether such a bias is compatible with other data, such as seismic data that suggest there are variations in physical and chemical properties in the lowermost mantle.

So much for the context. Channell and Lehman's contribution³ is that they have obtained and analysed high-resolution records from sedimentary cores in the North Atlantic. Not least, they used sediments with deposition rates of up to 12 cm kyr⁻¹ and thereby reduce the smoothing problem; in addition, they analysed several cores from the same general area to check for reproducibility.

When these results are compared with VGPs from previous measurements^{1,2} of sediment for the Brunhes/Matuyama reversal, we find support for the proposition that there may be substantial smoothing in many published sediment records. Moreover, the VGPs deduced by Channell and Lehman appear to reflect rapid variations in time, many of which seem to be evident in the other North Atlantic cores. So the primary source of the variation looks to be changes in Earth's magnetic field, rather than variations caused by rock magnetic problems. Unfortunately, such rapid variations probably make it unlikely that one can ever accurately

describe in detail the changes of the transitional field on a global scale by using spherical harmonic analyses; these analyses require a large number of globally distributed samples that are dated accurately enough to be sure that they represent the same snapshot of the transitional field.

Which of the three models described here do the new data best support? The authors say model 2, but this argument seems weak to me. There are not enough globally distributed data to describe the field for any one reversal transition, nor enough for different reversal transitions, to support or reject hypotheses that there are persistent patterns for VGP for different transitions.

Rather, I believe Channell and Lehman's achievement is to have shown that one can obtain valuable information on the transitional magnetic field from some sediments. They present a convincing case that there was large variation in the rates VGPs moved dur-

ing the two most recent reversals, and also demonstrate that data from sediments and lava flows can provide valuable complementary pictures of reversal transitions. Finally, they have provided a motivation for theorists to apply various filters to VGP data from different sediment cores and to develop alternative descriptions to spherical harmonics of transitional fields. □

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Evolutionary biology

Insights from the echinoderms

Eric H. Davidson

The evolutionary diversification of animal body plans has evidently devolved from alterations in the genetic regulatory programmes that control morphogenesis. This realization is now producing a tide of comparative descriptions of transcription-factor expression patterns, recorded at early stages of morphogenesis in various animals. Although these observations

are visually compelling, the same is not always true of the accompanying interpretations. These often depend on the assumption that regulatory gene expression, even if called into play at the very beginning of a developmental process, is likely to be dedicated to given morphogenetic functions far downstream, and that such functional linkages have remained immutable throughout evolution.

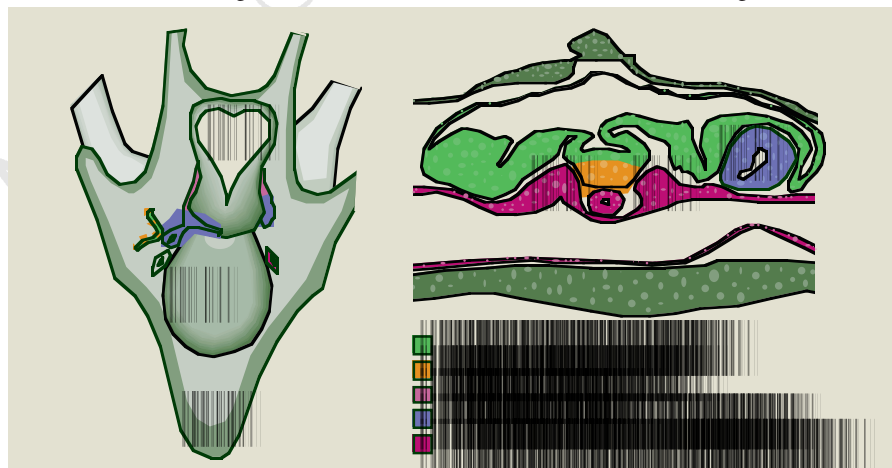


Figure 1 Origin of the main elements of the adult echinoid body plan in the imaginal rudiment. Lowe and Wray¹ have studied the expression of three transcriptional regulators in echinoderm development. They find that these factors are expressed in ways that are unique to the echinoderms, indicating that the same regulatory genes may have different functions during evolution. a, A larva, shortly after the completion of embryogenesis. The larval ectoderm (stippled) and the oesophagus (O) will be lost at metamorphosis, although the midgut or stomach (S) is retained. Shown in colour are the invaginating vestibule, including the neuroectodermal precursors that give rise to the central nervous system (green and orange), and the three bilateral coelomic domains from which the main mesodermal components of the rudiment arise. b, Cross-section of the rudiment, about mid-way through its development. In dark green at the bottom is the wall of the larval stomach, and at the top, the larval ectoderm. The ectodermal (green) and mesodermal (blue) elements of a podium (P) are included in the section; this, and the water vascular system, derives from the hydrocoels. (Figure adapted from ref. 17.)

There is much disagreement on this issue, which bears on the meaning of evolutionary homology in a developmental process and, indeed, on the very nature of developmental regulatory evolution. The paper by Lowe and Wray¹ (page 718 of this issue) now contributes to this discussion. They describe the expression of three homeodomain transcription factors — *distal-less*, *engrailed* and an *orthodenticle* orthologue — in the larval and juvenile development of sea urchins, starfish, sea cucumbers and brittle stars. These are four different classes of echinoderm.

The data of Lowe and Wray support two significant conclusions. First, morphogenesis of the definitive phyletic characteristics of the echinoderm body plan involves (and could depend on) the expression of *distal-less*, *engrailed* and *orthodenticle* in ways that are specific to the echinoderms. The main features of the modern adult echinoderm body plan are: fivefold radial symmetry (the sea cucumber is only secondarily bilateral); a calcite endoskeleton (test, spicules and/or spines); and a water vascular system, of which the tube feet (podia) are external locomotory projections. The authors found that all three of the genes are transcribed in fivefold radially symmetrical patterns during morphogenesis; that *distal-less* and *orthodenticle* are expressed during the early development of the podia; and, in brittle stars, that *engrailed* is specifically expressed in rows of ectodermal cells that overlie the gaps between the endoskeletal calcite plates. Thus, in echinoderms, these regulatory genes play some developmental roles that are distinct from those that they execute in either arthropods or chordates.

The second conclusion is that the usage of these transcription factors differs from class to class within the echinoderms. It follows that each transcriptional regulator was co-opted or recruited during evolution, for use in organizing new developmental functions, not only during the divergence of the radially organized echinoderms from their bilaterian deuterostome ancestors, but even during the divergence of the echinoderms themselves. Recruitment requires an underlying genomic event — the addition or alteration of *cis*-regulatory elements — which causes transcription of the gene in different spatial and developmental contexts. Such processes must have occurred in the *distal-less*, *engrailed* and *orthodenticle* genes, among many others, during the evolutionary radiation of the echinoderms.

The significance of this insight must be considered in terms of the general mechanisms by which morphogenesis of the main structural elements of the adult animal body plan is genetically controlled. In modern animals this is a stepwise process. Typically, it begins with the imposition of a new spatial regulatory state by means of expression of the genes that encode transcription factors in

a field of embryonic cells from which the structure will derive. These regulators activate genes that encode signalling systems and other transcription factors. In this way the domain is spatially subdivided into the ground-plan for the different parts of the future structure, all the while controlling the patterns of progressive cell growth within these subdomains. The genes that control cell-type-specific terminal differentiation programmes are called into play only after spatial patterning is well advanced.

Mechanisms of this nature have been uncovered in the morphogenesis of many arthropod and vertebrate structures; for example, in imaginal-disk patterning²⁻⁴; in the development of dipteran organ systems^{5,6}; and in vertebrate limb-bud development⁷, axial structures⁸ and various organs⁹⁻¹¹. A major feature is that the genes that encode patterning transcription factors in the initial stages of the process are usually used in various other regions of the forming body plan as well and, often, at several stages of each such process. An exclusive morphogenetic function cannot usually be assigned to such regulatory genes, because each is required for so many patterning processes. But there are also striking examples of the conserved use of particular regulators to build certain components of the morphology — the most prominent are the roles of a HOM-C/Hox complex in morphological diversification along the antero-posterior axis^{12,13}, and of *Pax6* in eye morphogenesis¹⁴. On the one hand, the multiplicity and variation in the developmental functions of given gene regulatory factors suggests that recruitment of patterning systems to new morphogenetic functions is common in evolution. On the other, the apparent conservation of morphogenetic function suggests retention of given developmental roles over long evolutionary periods. This opposition has spawned very different interpretations.

One of the most dramatic cases of recruitment seen by Lowe and Wray¹ is the expression of *distal-less* in the imaginal rudiment of a sea urchin (Fig. 1). The key ventral portions of the adult echinoderm body plan first develop in the imaginal rudiment. This forms from an invagination of the larval oral ectoderm (the vestibule) and a mesodermal coelomic sac, to which the invaginating ectoderm becomes closely apposed. The radial central nervous system, water vascular system and five initial podia are formed here.

Lowe and Wray¹ show that the entire inner portion of the invaginating vestibule is marked, from the beginning, by expression of *distal-less*. Not only is this an echinoderm-specific recruitment of *distal-less*, but it is a sea urchin (echinoid)-specific recruitment: other classes of echinoderm form their rudiments somewhat differently, and expression of *distal-less* was not observed in these. This is a good example of recruitment for an early

morphogenetic patterning function — the vestibular invagination is a transient structure, and its progeny will give rise to many diverse cell types.

Many of the special cases of recruitment reported by Lowe and Wray are in larval structures that, in these indirectly developing creatures, are destined to be jettisoned at metamorphosis. The embryonic processes by which these structures form consist, in general, of direct cell-type specification^{15,16}, and it is interesting that expression of the recruited gene always seems to occur in a single, differentiated cell type. Thus, *distal-less* is expressed in a specific form of larval mesoderm cell — the subtrochal cells of the arms of starfish larvae — and in a few neurons elsewhere. *Orthodenticle* is expressed only in the ciliated band cells of sea cucumber larvae, but not in ciliated band cells of other echinoderm larvae. Cell-type-specific expression occurs in the juvenile as well; for example, the expression of *engrailed* in neurons of brittle star arms. The class-specific use of the transcription factors studied by Lowe and Wray therefore occurs at diverse regulatory levels in the developmental process, and in the context of both larval differentiation and adult body-plan formation.

If recruitment is a general and dominant evolutionary process, then to reveal a true evolutionary homology between regulatory processes in two distant organisms we need a knife that cuts deeper than the mere observation of a transcription-factor expression pattern. A criterion that could be experimentally supported by looking at the relevant *cis*-regulatory domains might be as follows: in the absence of other information, to say that a transcription factor executes a homologous function during development it would be necessary to show that the gene encoding the factor is controlled by the same upstream regulators in the two organisms, and/or that the factor controls some of the same downstream genes. □

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Daedalus

Seeing round corners

As a car drives along a road, its powered wheels load the surface in tension, stressing it severely. So Daedalus began to devise a fibre-reinforced road, its concrete loaded with graphite fibres, or modern alkali-resistant glass fibres. But these days, glass fibre is an optical medium. This reminded Daedalus of the serious information limits of many roads, particularly the narrow, winding roads of rural areas. A motorist stuck behind a slow vehicle on such a road can never overtake safely: he cannot see round the bends to check that the other side of the road is clear. Daedalus now intends to give him this information, using the glass reinforcing fibres in the road.

His plan is to lay the fibres not continuously, but in specific lengths — a kilometre say. At their ends, the fibres will be bent upwards to terminate on the road surface. Each fibre will then accept light from one end, and re-emit it a kilometre down the road. A vehicle on the road, by blocking the light entering the fibres, would produce a visible 'road-shadow' at this distance.

If the kilometre fibres were laid in overlapping sections, spaced (say) at 100-m intervals, then the road-shadow of a moving vehicle would step along the road a kilometre ahead in 100-m strides: a spatial brightness modulation. A stationary observer, or a car moving the other way, would see a flickering road-shadow. The faster the closing speed, the faster would be its perceived flicker. If shorter fibres — 500 m, 250 m, and so on — were also laid in the road, and at correspondingly closer intervals, then as the closing vehicle got nearer, the road-shadow modulation would speed up. With proper spacing of the fibres, a driver watching the other lane of the road could read a true 'time of arrival' of the closest vehicle on that lane. Flicker is easily seen even on a very bright object (hence the flicker photometer and the blink comparator). So no matter how the road wound and dipped, the driver would know if anything was coming, and how soon it would arrive. By watching his own lane, he would even learn about the vehicles behind and ahead of him.

This system has all the virtues. It is cheap, simple, lasts indefinitely, and has no electronics. It will work just as well in the dark, when the road-shadow will be replaced by a road-glow from the oncoming vehicle's lights. It is simple to interpret. It even strengthens the road.

David Jones

Two sets of human-tropic pig retrovirus

Advances in controlling immunological rejection have raised the possibility that pigs could be used as a source of organs and tissues for transplantation into humans^{1,2}. However, the report that one pig kidney cell line, PK15, produces C-type retroviruses capable of infecting human cells³ has reinforced fears over the potential risks of viral infections associated with xenotransplantation^{4,5}. Further support for these fears comes from the discovery of two different classes of porcine endogenous proviruses (PERVs), capable of infecting human cells, in PK15 cells as well as in a variety of normal porcine tissues.

The envelope gene is the principal determinant of retroviral tropism⁶ and so may be important in determining which PERVs are capable of infecting human cells. We set out to clone this region from the proviruses expressed by PK15 cells using primers from the well conserved pol-RT region of PERV³. We used 3' rapid amplification of cloned ends (RACE)⁷ to clone PERV envelope sequences from the pig cell line PK15 and a human cell line named 293 infected with virus from PK15.

Amplification products of around 6 kilobases (the expected size of products from the primer used to the poly(A) tail of C-type retroviruses) were isolated and cloned. Analysis of the clones obtained revealed two classes of sequence (PERV-A and PERV-B), with open reading frames of 660 and 657 amino acids, respectively (Fig. 1). On the basis of their position in the viral genome and their sequence, the clones seem to correspond to the viral envelope.

Clones with sequence corresponding to both classes of envelope were obtained from PK15 cells, although 29 of 32 clones were characterized by restriction mapping as PERV-A and only three as PERV-B. Of 12 clones characterized from cell-line 293 infected with PK15 virus, all were of the PERV-B class. The two predicted envelope proteins are very similar in their transmembrane region but show greater differences in the predicted surface region. The greatest diversity occurs in the regions which correspond to regions of the murine leukaemia virus responsible for determining receptor binding⁸. The extent of the variation in these regions of the two PERV classes suggests that they will use different receptors.

Comparisons of the transmembrane region of the envelope protein with sequence databases show that the PERVs belong to the mammalian C-type retrovirus family. PERV-A and PERV-B showed 92%

amino-acid identity to one another and 63–66% identity to gibbon ape leukaemia virus, feline leukaemia virus and Friend murine leukaemia virus.

The identification of sequences specific to the two classes of PERVs found in PK15 cells allowed us to design primers which

would specifically amplify complementary DNA from each class (PL170–173; see Fig. 2 legend). We used these primers to determine the expression pattern of the two classes in a range of pig tissues and human 293 cells infected with PERV-PK. In all pig tissues tested (heart, spleen, kidney), we



Figure 1 The envelope proteins of PERV-A and PERV-B. Identical amino acids are shown with a dot and gaps are indicated with a dash. The positions of the regions of the protein inferred to be important for host range determination⁸ on the basis of comparisons with murine leukaemia virus are shown. The predicted cleavage site between surface and transmembrane is marked with an arrow. Nucleotide sequences have been deposited in the EMBL Nucleotide Sequence Database with accession numbers Y12238 and Y12239.

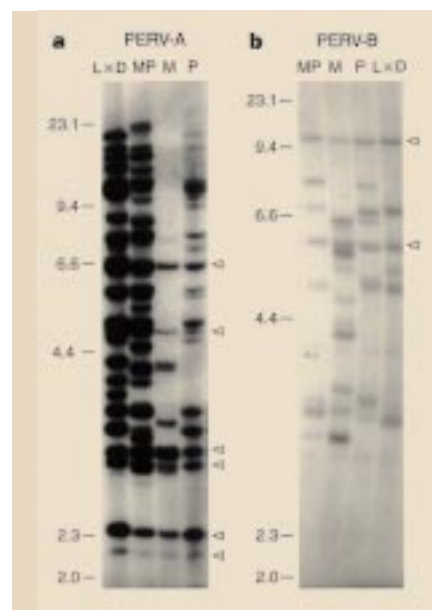


Figure 2 Southern analysis of the number and genetic distribution of PERV proviruses. **a**, PERV-A: *Nsi*I-digested genomic DNA hybridized with labelled PL170 (TGGAAAGATTGGCAACAGCG)/PL171 (AGTGATGTTAGGCTCAGTGG) PCR-amplified PERV-A plasmid DNA. **b**, PERV-B: *Bam*HI-digested genomic DNA hybridized with labelled PL172 (TTCTCTTTGTCAATTCGGG)/PL173 (TACTTTATCGGGTCCACTG) PCR-amplified PERV-B plasmid DNA. Agarose gels were dried and hybridized as in ref. 10. The ability of the probes to distinguish the two classes was demonstrated by hybridization to Southern blots of plasmid clones of each class (not shown). DNA samples were prepared from Landrace x Duroc (L x D), Meishan (M), and Pietrain (P) animals and from the mini pig kidney (MP) cell line. The restriction enzymes chosen yield characteristic fragments containing 3' viral sequences joined to unique flanking sequences for each provirus. Proviruses that appear to be shared between all samples are indicated with arrows.

detected expression of both classes of PERV, showing that both PERV-A and PERV-B are transcribed in normal tissues as well as in tissue culture cells (data not shown). We also found expression of both classes of PERV in cell-line 293 infected with PERV-PK, but not in uninfected 293 cells. Preliminary host range experiments using recombinant *env* genes confirm that both PERV-A and PERV-B are capable of infecting human cells.

The differences between the two envelope genes allow the preparation of specific probes for Southern hybridization analysis. Using strategies developed for the genetic analysis of endogenous murine proviruses⁹, we have started to analyse the proviral content of different breeds of pig, because one way to reduce the risk of PERVs for xenotransplantation would be to identify or breed source animals that do not express replication-competent PERV.

Hybridization analyses (Fig. 2) show that both classes of PERV are inherited in a range of pig breeds, and so are likely to be present in those pigs that have been genetically modified to be used as sources of organs for xenotransplantation. PERV-A proviruses are present at between 10 and 23 copies and PERV-B between 7 and 12 copies. Some polymorphism was seen between the individuals used, which would be important if breeding were to be used to try and eliminate proviruses; however eight proviral fragments were common to all pigs tested.

Although we do not yet know which proviruses are capable of yielding infectious virus, the number of proviruses present suggests that the breeding of virus-free pigs, if at all feasible, will represent a complex task.

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Rock-eating fungi

Weatherable minerals under many European coniferous forests contain a network of numerous tubular pores, formed by organic acids exuded by fungi. We believe that symbiotic mycorrhizal hyphae translocate dissolved minerals from the isolated micropores directly to their host plants, bypassing competition for nutrient uptake by other organisms. The discovery of this pathway challenges current ideas about nutrient uptake from the bulk soil solution and criteria for critical loads of acidic deposition on forests.

Pores of width 3–10 μm , which sometimes contain fungal hyphae, are widespread in feldspars (Fig. 1, 2) and hornblendes in



Figure 1 Thin-section micrograph in cross-polarized visible light with 550-nm retardation (gypsum plate), showing an alkali feldspar from a podzol E soil horizon (yellow) with micropores (purple) and 5- μm -thick fungal hyphae (brown).

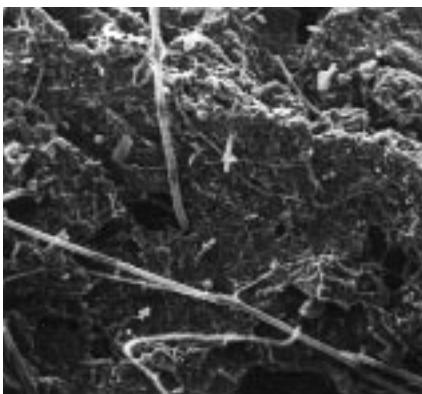


Figure 2 Scanning electron micrograph, showing 4–6- μm -thick hyphae entering a calcium feldspar at a granite surface near Lunsen, Sweden.

podzol E soil horizons and granitic bedrock under *Pinus sylvestris*, *Picea abies* and ericaceous shrubs in Sweden, Finland, Denmark, the Netherlands and Switzerland. Micropores are rare in deeper podzol horizons and in acidic brown forest soils (Cambisols). We believe that the pores were formed by mycorrhizal or saprotrophic fungi, which exude organic acids¹ from their hyphal tips. Such acids enhance mineral weathering by forming complexes with aluminium.

We found micro- to millimolar concentrations of succinate, citrate, oxalate, formate and malate in Swedish bulk soil solutions. Concentrations are likely to be higher near hyphal tips inside the pores. Fungal hyphal tips producing millimolar concentrations of these acids could dissolve calcium-rich plagioclase feldspars to form pores at rates of 0.3–30 $\mu\text{m yr}^{-1}$ (ref. 1). From total-element soil analyses, we calculated losses by weathering in one Swedish soil (assuming zirconium to be inert²), and infer that ~150 m of pores are formed annually per dm^3 of E horizon, and that about 10^7 hyphal tips are eating their way through sand grains at any one time. Each feldspar grain would contain several active hyphal tips, but less than 1% of all pores would contain hyphae. These values are consistent with our micromorphological observations in podzol E horizons.

In Sweden, the abundant hyphae that occupy podzol E horizons and the dense mycelial mats on granitic bedrock surfaces are predominantly ectomycorrhizal. We have observed direct hyphal connections between rock surfaces and plant roots colonized with the ectomycorrhizal fungus *Suillus granulatus* and *Piloderma croceum*. We therefore expect that our DNA-based identification of fungi on rock surfaces and the tracing of weathering products in fungi will show that ectomycorrhizal fungi are the main organisms mediating pore formation.

Nutrient supply by mycorrhiza from inside minerals upsets conventional ideas of mineral nutrient uptake from the bulk soil solution. Acid atmospheric deposition has depleted exchangeable basic cations³ and increased concentrations of dissolved, inorganic, phytotoxic Al^{3+} in soils⁴. Organically complexed aluminium released during pore formation by fungi is less phytotoxic⁵. Low ratios of dissolved $\text{Ca}^{2+}/\text{Al}^{3+}$ are particularly detrimental to tree roots⁶ and critical values of $\text{Ca}^{2+}/\text{Al}^{3+}$ in nutrient solution experiments have been used to calculate critical loads of acid atmospheric deposition⁷. If trees can access Ca^{2+} and Mg^{2+} from inside mineral grains, Al^{3+} in acidified soil solutions may be irrelevant to the uptake of calcium by roots, and the calculated critical loads of acidic deposition may be invalid.

Our findings question the need to add lime to acidified podzolic forest soils in Sweden, a process that is often thought necessary⁸. A mechanism by which mycorrhizal

hyphae can bypass the acidified soil to reach minerals may help explain why forest productivity across Europe has not decreased⁹, in spite of recent excessive soil acidification.

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Sex in giant squid

Recent captures of two female giant squid (*Architeuthis*) off southern Australia have provided the first record of a mated female specimen of these almost mythical deep-sea creatures. We found sperm packages (spermatophores) embedded within the skin of both ventral arms of the larger of the two specimens. It seems that male giant squids may use their muscular elongate penis to 'inject' sperm packages under pressure directly into the arms of females.

Both animals (Fig. 1a, b) were captured live during commercial fishing trawls off Tasmania at depths of between 500 and 1,000 m. Most of the skin of the largest animal (Fig. 1b, 220 kg, mantle length 2.4 m, estimated total length 15 m) was intact, and we discovered the embedded spermatophores in the skin on both ventral arms. We found a section of sperm cord protruding from a small shallow wound (diameter <1 cm) on the right ventral arm, 83 cm from the mouth. Dissection around this site exposed the remains of at least three sper-

matophores stored in the skin, apparently radiating (up to 8 cm) from a single point of entry (Fig. 1c, d).

Females of many cephalopod species store sperm in specialized seminal receptacles, sometimes for several months¹. In different species these receptacles are on the external mantle, head, arm-crown, arms, around the mouth, within the mantle cavity or within the reproductive tract^{1,2}. No such receptacle has been reported for the 20 or so female *Architeuthis* specimens found to date³. The spermatophores in our specimen were embedded in the skin at the same level on both ventral arms (and in no other arms), suggesting deliberate placement of these sperm packages. We found no additional spermatophores elsewhere in the skin or within the mantle cavity. The failure until now to find sperm stored in the skin may be a result of skin loss, common in previously found specimens (Fig. 1a).

Sperm storage in the skin occurs in other much smaller squids^{4–6}, where spermatophores are stored in healed elongate wounds on the arms or mantle. Males may use their beak or the sharp scythe-like hooks found on the tentacles and/or arms of certain species to form these wounds^{2,7}. These squid possess a long muscular penis and lack the modified arms used by males of most other cephalopod species to pass spermatophores to females. This morphology suggests that these smaller species use the penis to insert spermatophores directly into the female's wounds.

Little is known of *Architeuthis* males in southern oceans — all previous specimens from these waters have been female⁸.

Northern Hemisphere males possess large spermatophores (11–20 cm long), a greatly elongated muscular penis (up to 92 cm long, emerging up to 55 cm beyond the mantle cavity⁹) and only slight modification of the ventral arm tips, as two rows of low 'pads'^{3,9,10}. This configuration suggests that male giant squids also use the penis to transfer spermatophores directly to the female. The absence of elongated wounds, along with the multiple spermatophores found adjacent to a single small opening in our specimen, indicates that the muscular penis might 'inject' the spermatophores into the skin, potentially under hydraulic pressure. *Architeuthis* spermatophores are enveloped in a gelatinous coating^{9,10}, not reported in any other cephalopods, which may act as a lubricant to aid injection into the skin of the female.

Accidental injection may have occurred in a male *Architeuthis* caught off Norway in the 1950s⁹ in which spermatophores were found embedded in the skin of several arms and the mantle. Another male may have injected the spermatophores while attempting to impregnate a female, accidentally 'riveting' a co-suitor. Alternatively, this male may have literally 'shot' himself in the foot.

Our mated female giant squid was submature with an ovary of ~3 kg, occupying less than 20% of the mantle cavity and consisting of hundreds of thousands of undeveloped eggs. The discovery of sperm in this animal suggests that female *Architeuthis* can store sperm from submature stages, as reported in other cephalopods¹, perhaps reflecting that encounters with male giant squids are infrequent at these dark depths.

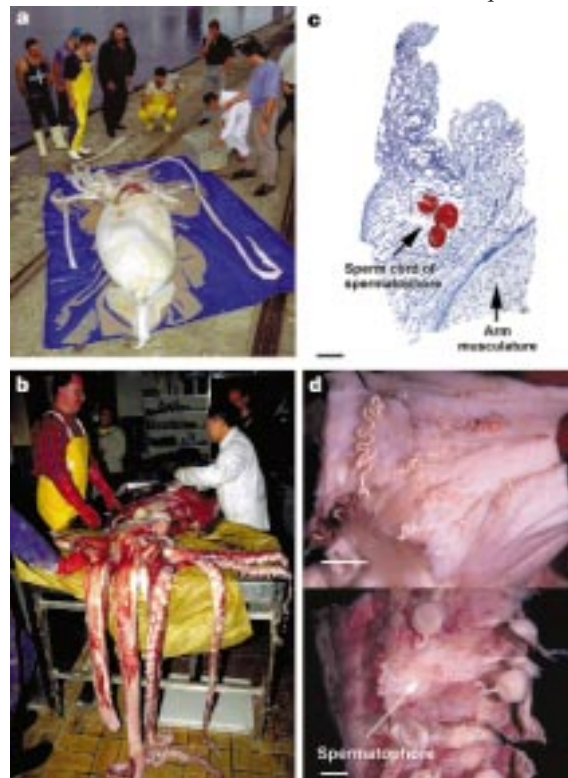


Figure 1 Mated giant squid. **a**, First of two female giant squid captured in commercial fishing trawls off Tasmania, Australia, in 1996. The majority of the skin is missing. **b**, Second specimen. A 220-kg submature mated female with skin largely intact. Spermatophores were embedded under the skin of both ventral arms. **c**, Transverse section of left ventral arm of the 220-kg female showing spermatophores embedded within the epidermis (scale bar, 1 mm). **d**, Spermatophores within the skin of the right ventral arm, in the aboral surface (top) and amongst suckers on oral surface (bottom). Scale bars, 10 mm. Photographs, D. Paul; histology, B. Abaloz.

As spawning events have never been witnessed for 'skin-storing' squids (including *Architeuthis*), it is not known how females access sperm to fertilize their eggs. The suckers or beak may be used to peel open the skin covering the spermatophores, or the sperm may migrate to the surface on hormonal or chemical cues. Alternatively, the female's skin might degrade on spawning, exposing the embedded sperm stores.

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Homeotic transformation in *Drosophila*

Fossil records indicate that insect wings are pleural appendages, primitively present on three thoracic and the nine abdominal segments as wings and winglets, respectively¹. Homeotic proteins are generally considered to be the ontogenic switches governing the choice of developmental pathway of such appendages². Here we provide examples of regulatory changes in developmental genes leading to a homeotic transformation in the ectopic wing tissue of prothoracic or metathoracic segments in *Drosophila melanogaster*, which lead to the development of wings rather than halteres.

Of the two homeotic clusters in *Drosophila* (*Antennapedia* and *Bithorax*), only the *Antennapedia* (*Antp*) gene is expressed in segments from which the wings and halteres arise. However, *Antp* is not required to form thoracic wing primordia or the adult wings³, indicating that ancestrally, wings probably arose in all segments without homeotic gene involvement (the homeotic ground state)^{3,4}. Furthermore, regulatory interactions between homeotic proteins and the developmental genes involved in appendage formation seem to have been important evolutionary innovations in the diversification of arthropod body plans and appendages⁵. If this is true for the evolution of insect wings, then changes in homeotic or developmental genes should result in

homeotic transformations.

The *engrailed* (*en*) gene encodes a homeodomain-containing transcription factor which establishes the posterior identity of embryonic and adult segments⁵. The *wingless* (*wg*) gene encodes a member of the Wnt family of signalling molecules which regulates several developmental pathways in embryos and imaginal discs⁶. We ectopically expressed *en* from a constitutive promoter in cell clones generated by the *flp*-out cassette⁷.

We identified clones expressing *en* on the wing and haltere by the presence of forked (*f*) bristles. As expected, those in the anterior compartment of wings showed characteristics of the posterior compartment and organized new anterior compartments in front of them (see also ref. 7). But all of the 65 clones that we observed induced transformations of halteres to wing in anterior haltere compartments, whereas the posterior haltere compartments maintained their normal structure and remained associated with transformed wing (Fig. 1a). Because *Ultrabithorax* (*Ubx*) mutations transform haltere primordia to wing primordia⁸, it is likely that this transformation of halteres into wings, or of wings into halteres by the absence of *en* function⁹, may be

due to negative regulation of *Ubx* by *en*¹⁰.

To examine the role of *wg*, we used different hypomorphic alleles, such as *wg^P*, *wg¹*, *wg^{CX3}*, *wg^{CX4}* and *wg^{lacZ}* (refs 11,12), some of which are regulatory mutations and, except for *wg¹*, are homozygous lethal at different developmental stages. Several heteroallelic combinations are viable and we analysed these for defects or transformations at emergence or at pharate adult stages. Heteroallelic combination of *wg^P* with *wg¹*, *wg^{CX3}*, *wg^{lacZ}* or *wg^{CX4}* induced the development of wings in the prothoracic (T1) segment. About 30% of the 200 *wg^P/wg^{lacZ}* flies examined had new T1 wing-like structures, ranging from rudimentary to well-formed (Fig. 1b).

In the *wg^P/wg¹* heteroallelic combination, T1 wings were seen in 25% of individuals whereas the mesothoracic (T2) wings and metathoracic (T3) halteres developed variably. In other heteroallelic combinations such as *wg^P/wg^{CX3}* or *wg^P/wg^{CX4}*, 25% showed T1 wings but the T2 wings and T3 halteres were always absent. Presence of T1 wings even when normal T2 wings and T3 halteres were absent confirms a segment-specific differential expression and regulation between the homeotic and developmental genes.

The heteroallelic combination *wg^P/wg^{CX3}* also showed a varying degree of transformation of T3 legs into halteres in 10% of individuals (Fig. 1c), ranging from graded removal of the distal segments (tibia and tarsus), through distortion of the femur and graded removal of leg bristles, to transformation of the coxa and trochanter into the first and second segments of the haltere.

These homeotic transformations in *Drosophila* thoracic discs caused by the misexpression of developmental genes like *wg* or *en* indicate that altered regulation of developmental genes was important in the evolution of body plans and the elaboration of new structures.

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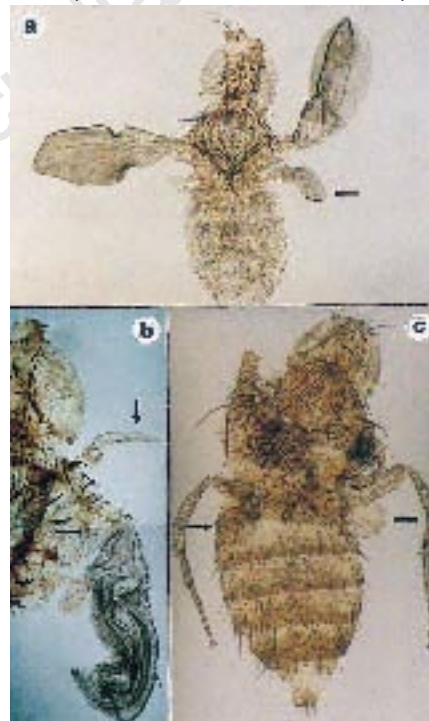


Figure 1 Homeotic transformations in *Drosophila*.

a, Ectopic expression of *En* in a *Tuba1 > en* somatic clone in the anterior compartment of the right haltere (arrow) caused its transformation into a wing. The left haltere is normal. **b**, A prothoracic wing (arrow) in a *wg^P/wg^{lacZ}* pharate adult fly. Normal T2 wings and T3 halteres are also seen. **c**, Transformation of a metathoracic leg to a haltere (arrows) in *wg^P/wg^{CX3}* pharate adult fly. The common phenotype of *wg^{CX3}* mutants, absence of T2 wings and T3 halteres, and duplication in the thoracic segments, is also seen.

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Betrayers of a global truth

The Heat Is On: The High Stakes Battle over Earth's Threatened Climate

by Ross Gelbspan

Addison-Wesley: 1997. Pp. 278. \$23, £17.95

Tim O'Riordan

Imagine you are a leading scientist, about to address a press and congressional briefing in Washington, comforted in the soundness of your case by the most rigorous and global peer-review. You are to present the findings of the scientific working group of the Intergovernmental Panel on Climate Change (IPCC). Your analysis points to inescapable evidence that the human hand is altering the global climate, possibly for many centuries to come, particularly through emissions of carbon dioxide from fossil fuels.

Without warning you are attacked by lawyers with all the aggression and discourtesy of the courtroom. Your family is harassed by late-night telephone calls, and your own life story and scientific history are subject to mischievous distortion in planted stories published throughout the United States. There is a dedicated campaign to prove that you doctored your colleagues' conclusions so that the outcome looked

faulty and deliberately concocted.

This could be the stuff of fiction and would make an excellent television drama. But this was no movie. This is what happened to the lead scientific editor of the IPCC report that produced the most scrutinized and debated scientific analysis that the standard peer-review procedures of science can deliver. Ross Gelbspan has produced a marvellously readable but devastatingly candid account of the brutal politics of debunking the scientific method by the opulent vested interests of the fossil-fuel lobby.

There is a class of American journalist who can make environmental politics sing with the lyricism of captivating narrative. Gelbspan is one, another is Bill McKibben, who eulogizes this book on the dust cover, and a third is Philip Shabacoff, who would surely agree. Scientists really ought to read this book, and so too should any student of environmental politics and climate-change negotiations. For here is the inside story, well researched and faithfully presented, of the vicious financing of the 'contrarians', namely the climate-change sceptics, financed by the fossil-fuel lobbies, who masquerade as university professors, publish no peer-reviewed papers, and yet get the ear of Congress.

Make no mistake about it, the science may have concluded that climate change is induced by humans and that the outlook for vulnerable peoples and ecosystems is awesomely threatening. But the 'science' the US Congress and business leaders are listening to is the pseudoscience that Gelbspan so articulately and incisively examines.

Why is this? Part of the answer obviously lies in raw political lobbying. The United States may be a democracy, and an open one at that, but its politics are overwhelmed by lawyers, lobbyists and high financial stakes. Part of the answer also lies at the feet of science itself. Scientists are trained not to transgress into the world of judgement and political bickering. One can readily understand why. However, as Gelbspan also explores, scientists are privately alarmed at the possibility of catastrophic convulsion in climate change, and at the long-term implications of a warming trend combined with unpredictable rainfall. But they cannot come out publicly and say so because the evidence is not strong enough. The scientist is therefore inherently conservative in the analysis and openly honest about the uncertainties.

But, in the blood-letting world of oppositional politics, this silence is filled with the clamour of persuasion. The lay politician succumbs, buffeted by demands for an open, competitive economy and the freedom to drive cars on ludicrously low gasoline prices. The science is not insistent enough to help the legislator withstand the American dream of material progress and economic security.

At Kyoto, Japan, in December, the next meeting of the conference of the parties to the United Nations Convention on Climate Change is due to produce a protocol limiting greenhouse gas emissions by some identifiable percentage by 2010. It is no wonder that the United States will go to that meeting without a significant target on offer. The best that can be hoped for is a reduction of a few per cent on 1990 levels by 2010. This is far below the wishes of the scientific community, to say nothing of the billions for whom such a token gesture spells great misery. The United States seems politically and ideologically incapable of coming to terms with the moral and inequitable aspects of global climate change. Even the best and most comprehensive scientific endeavour will not alter this perspective.

Gelbspan tells us why and, in passing, indicates in raw italicized passages just how the world might look in the next century if the scientists continue to be outflanked. He looks to the insurance industry for a future ally with the scientists, but that is a dubious conclusion. It is more likely that the industry will introduce a tough regime of higher

Exploring Earth's mountains of fire



A civil defence helicopter is dwarfed by a 400-metre-high lava fountain during Kilauea Volcano's Puu Oo eruption in Hawaii in 1984. The picture appears in the third edition of *Volcanoes* by Robert Decker and Barbara Decker (W. H. Freeman, \$19.95 (pbk)), who provide a brief, authoritative introduction to these natural phenomena, in vivid prose: "Volcanoes assail the senses. They are beautiful in repose and awesome in eruption; they hiss and roar; they smell of brimstone. Their heat warms, their fires consume; they are the homes of gods and goddesses." The new edition contains descriptions of recent eruptions and findings, as well as a list of Web sites carrying news of volcanic activity around the world.

premiums, along with many more escape clauses. As a result, the vulnerable will decreasingly be protected by a commercial sector that cannot afford to be charitable. Crisis nongovernmental organizations will be left to pick up the pieces while governments wring their hands in passive culpability.

The inescapable conclusion is that climate-change politics are embedded in institutions whose survival is dependent on an economy and ideology that create climate change. Gelbspan reveals this tragic truth with candid clarity. Don't look to Kyoto for salvation. But do look to a more aggressive and politicized science that draws on the moral underpinnings of humanity for its message. □

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From cats to computers

Quantum Technology

by Gerard Milburn

Unwin: 1997. Pp. 188. £7.99 (pbk)

An Introduction to Quantum Theory

by Keith Hannabuss

Oxford University Press: 1997. Pp. 380. £35, \$65

Interpreting the Quantum World

by Jeffrey Bub

Cambridge University Press: 1997. Pp. 298. £35, \$49.95

Alastair I. M. Rae

Gerard Milburn's *Quantum Technology* aims to explain and indeed celebrate the practical achievements that have emerged from our greater understanding of quantum physics in recent years. He manages to do this without employing a single mathematical expression, but instead uses a skilful combination of verbal arguments and diagrams.

The fundamental ideas of quantum mechanics are explained in a chapter on "Quantum Roulette" in which the quantum principle of combining probabilities is compared and contrasted with the laws of classical probability. After this follow five chapters, each on a different aspect of the new technology.

The use of Doppler cooling and magnetic traps to isolate and hold still single atoms is the subject of one of these. Initially, one is tempted to wonder what is particularly quantum about this example — certainly, it relies on the existence of atomic energy levels and radiation pressure and all these are quantum to some extent, but no more than many everyday phenomena (sodium street lamps, for example). But Milburn demonstrates how an ability to control atoms at this level can lead to the performance of atom

Celebration of light and landscape

Many rarely seen images by the renowned Californian photographer Ansel Adams are reproduced in a new book edited by Andrea G. Stillman.

"One of the first things I noticed about California was the quality of the light," writes Stillman. "It was almost palpable, as if you could reach out and touch it. It was light that inspired Ansel to photograph, and it was his preternatural feeling for light that made his work approach the sublime. He worked almost exclusively at dawn or sunset; the rest of the time he found the light too flat, the forms of the landscape dull and uninteresting." The pictures in *Ansel Adams California* (Little, Brown, \$50) are accompanied by writings about the state by classic and contemporary authors from Robert Louis Stevenson to Jack Kerouac.



interference experiments and the potential, at least, to use these to do lithography and construct devices at an atomic length scale.

The last and perhaps most exciting chapter is on the "Quantum Computer". Milburn provides a crystal-clear description of the "square root of NOT" which is an essential step along the way and explains how one such device might control the other, although at this point he talks about measurements that are apparently reversible — surely a quantum heresy!

I think I just about understand how a quantum computer could be used to perform operations that are impossible in principle in any conventional computer — which is an advance over anything else I have read in this area, including articles by David Deutsch who was responsible for the invention of the principle.

More detail about the type of technological barriers to the realization of a quantum computer would have been welcome and, although it would have been a departure from his technological theme, Milburn could also have indicated how the parallel calculations performed by a quantum computer can be interpreted as circumstantial evidence for the many-worlds view of quantum mechanics.

Keith Hannabuss's *An Introduction to Quantum Theory* is a textbook based on lectures given to second- and third-year mathematics students at the University of Oxford, England.

The first five chapters introduce wave mechanics and include the solution of the Schrödinger equation for the hydrogen atom and quantum-mechanical tunnelling. The more formal theory is then developed, assuming a prior knowledge of vector space theory; fairly standard treatments of topics such as angular momentum and many particles follow, and the book ends with a chapter on the Dirac equation.

Although the author claims that his treatment is less than rigorous, it is considerably more advanced than that taught to many undergraduates. The coverage is quite conventional on the whole and comparable with that in Dirac's own classic text, but Hannabuss brings his treatment up to date with sections on coherent states and squeezed light. An interesting, but rather difficult, chapter on symmetry in quantum theory covers material more commonly met in postgraduate courses.

Students who master the contents of this book as undergraduates would have considerable advantages over many of their contemporaries if they moved on to research in theoretical or mathematical physics.

As is common nowadays, Hannabuss's book contains a chapter on "Measurements and Paradoxes". This includes a treatment of Bell's theorem and the Schrödinger cat problem, but somehow I don't think the author's heart is really in it.

This is particularly true of the end of the chapter where his criticism of the decoher-

ence interpretation of quantum mechanics omits the central criticism that even if there are no correlations, the standard probability interpretation still has to be put in 'by hand'. He also fails to explain why it has led many people to take the Everett relative state theory more seriously, realizing that the central feature of this theory is not so much the splitting of the Universe as its potential for reunification in a future interference experiment.

No-one could accuse Jeffrey Bub of not taking the conceptual problems of quantum mechanics seriously. His authoritative book *Interpreting the Quantum World* makes no attempt to address a general audience, but consists of deep and detailed consideration of most, if not all, current thought in this area.

Many technical results are described and proved in detail. Bub clearly understands the Everett theory, but does not accept it. He explains David Albert's argument leading to apparent inconsistencies between different predictions of the answer an observer (Eve) would make to a questioner (Adam) about whether she has a definite belief about the result of the observation of the polarization state of a photon.

But I do not believe that Bub and Albert have properly taken on board the effects of decoherence, which explains why the classical states provide a preferred basis for this description. It seems to me that this inevitably makes it impossible in practice for Adam to address his question to the whole of Eve's mental state rather than to the branch of it in which he himself exists. I wonder whether similar considerations will not also form an insuperable barrier to the practical realization of a quantum computer.

It is probably no surprise to those who know about Bub's work that he ends up defending hidden-variable theories similar to those invented by David Bohm, with whom Bub started his research career. But I detect no trace of the "implicate order" beloved by the later Bohm, so I hope that Milburn would include this book in the subset of such texts that he describes as "quite excellent" in contrast to others that "invoke time-worn mysticism, both western and eastern". □

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Related books

Mathematical Undecidability, Quantum Nonlocality and the Question of the Existence of God edited by Alfred Driessen and Antoine Suarez. Kluwer, \$160, £95. Contains Paul Davies' acceptance lecture for the 1995 Templeton Prize.

The Message of the Atoms: Essays of Wolfgang Pauli and the Unspeakable by Kalervo V. Laurikainen. Springer, \$42, £26. Philosophical basis and implications of the Copenhagen interpretation of quantum mechanics.

At a glance

Excellent ★★★★★ Good ★★★★ Fair ★★★ Poor ★

Radioactive and Stable Isotope Geology

by H.-G. Attendorn and R. N. C. Bowen
Chapman and Hall: 1997. Pp. 522. £95, \$174.95

Isotopic methods today crop up in every branch of modern Earth sciences and can elucidate countless processes that have shaped the Earth throughout its history, as well as making possible the dating of geological events. Here is a conscientious attempt to summarize the general principles of isotope geology as well as methods and techniques available for radioactive isotope dating, stable isotope abundance studies in the biosphere, and isotopic studies of terrestrial and planetary lithospheres.

The book is updated from an earlier version published in 1988. Because of the wide coverage, each section can provide only a basic introduction to principles and applications, and not all sections show the same critical insight. The reference list is not particularly exhaustive or up to date.

The descriptive density of the text and the overall paucity of illustrations may motivate prospective researchers to seek out more specialized textbooks and review articles.

The book is therefore recommended primarily for science libraries and laboratory reference shelves.

Stephen Moorbath Department of Earth Sciences, University of Oxford, Parks Road, Oxford OX1 3PR, UK.

Range	★★★★★
Depth	★★★★
Accuracy	★★★★
Up-to-dateness	★★★
Accessibility	★★★★
Style	★★★★

The Colours of Life: Introduction to the Chemistry of Porphyrins and Related Compounds

by Lionel R. Milgrom
Oxford University Press: 1997. Pp. 249. £22.50, \$95

"Porphyrins: molecules for all seasons" comes to mind as an alternative title for this book. The thesis that porphyrins permeate nature is sustained by seven chapters ranging from the origin of the Solar System and abiotic synthesis of porphyrins to their use in cancer therapy and possible application in molecular electronics. What other book would cover subjects as diverse as Kant-Laplace theory, anti-aromaticity and the Peierls transition?

The porphyrin-oxygen duet is the book's centrepiece. First we learn that the high oxidation potential available in PSII chlorophyll made possible the evolution of oxygen (a terrific pollutant which must have brought an end to much of early life).

Then we see how oxygen handling by haemoproteins made the full energy of reduced

Paleontological Events: Stratigraphic, Ecological, and Evolutionary Implications

edited by Carlton E. Brett and Gordon C. Baird
Columbia University Press: 1997. Pp. 604. \$65, £52

Over the past 150 years, many groups of fossils have been used to establish a detailed biostratigraphy for Phanerozoic time. Biozonal schemes can achieve temporal subdivisions as short as 0.5–1.0 million years for strata as old as the Silurian period (417–443 million years ago) using the extinct graptolites. But is this the best that can be achieved, given the nature of the fossil record?

This multiauthored volume shows that renewed understanding of short-term 'catastrophic' episodes has recently opened the way to the possibility of such geologically 'instantaneous' events being recognized by fossil biomarkers.

Storms, earthquakes and volcanic eruptions all produce particular effects on land, different ones on the continental shelf and yet others in deeper marine basins.

The essays show how an understanding of the processes and effects of short-term events in different depositional environments are helping to pinpoint such events in Lower Palaeozoic rocks in North America.

Douglas Palmer 31 Mawson Road, Cambridge CB1 2DZ, UK.

Range	★★
Depth	★★★
Accuracy	★★★
Up-to-dateness	★★
Accessibility	★★★
Style	★★★

carbon compounds available so that aerobes could evolve. And, finally, we discover that the photophysics of singlet oxygen sensitization by porphyrins which nature struggled to suppress in photosynthesis is used, ironically, to our advantage in photomedicine.

The chemistry along the way is elaborate, engaging and presented with unusual insight.

What is the price for exploring porphyrin chemistry on geological to photophysical timescales in 249 pages? Not too high: certain details about photosynthesis are confused and the latest information in the final chapter on "Porphyrins for the future" is about four years old.

Thomas A. Moore Department of Chemistry and Biochemistry, Arizona State University, Tempe, Arizona 85287-1604, USA.

Range	★★★★
Depth	★★★★
Accuracy	★★★★
Up-to-dateness	★★★★
Accessibility	★★★★
Style	★★★★

In retrospect chosen by Philippe Janvier

Les chiens aboient ("The dogs bark")

by Herbert Wild
(1926)

This rare book had fallen into oblivion, but gained renewed interest with the recent "Gupta case" (see *Nature* 341, 16; 1989). It is a largely autobiographic novel about a similar case of palaeontological fraud — so far unresolved — which shattered French geology between 1917 and 1922.

Herbert Wild, the author, is the pseudonym of Jacques Deprat (1880–1935), a talented French geologist accused of "salting" sites in Yunnan and northern Vietnam with fossil trilobites from Europe. Deprat was fired from the Geological Survey of Indochina in 1920 and banned from the Société Géologique de France. Instead, he became a successful writer, publishing 13 novels between 1924 and 1937. (One of them, *Le Colosse endormi* ("The sleeping Colossus"), a prophetic book about China, won the Grand Prix of the French of Asia, competing against André Malraux's *La voie Royale*.)

The Deprat case was revisited in detail in 1990 by a French geologist, Michel Durand-Delga, who became convinced that Deprat was innocent. Yet no indisputable evidence for his innocence has ever been found, and the palaeontologist Jean-Louis Henry, an authority on trilobites, maintains that at least three of the suspected specimens were actually from Europe, presumably from Bohemia.

Deprat studied for his thesis at the Muséum National d'Histoire Naturelle in Paris under the supervision of the mineralogist Alfred Lacroix. After a stint as assistant professor in geology in Besançon, and on the recommendation of Pierre Termier, he became director of the Geological Survey of Indochina in Hanoi in 1909, at the age of 29.

Between 1909 and 1917, Deprat mapped much of southern China and northern Vietnam and published memoirs on the geological structure of Indochina. He reorganized the Geological Survey of Indochina, turning it into a centre for real scientific research (as opposed to routine engineering and mapping) and regularly published its scientific proceedings. Initially, his only collaborator in palaeontology was Henri Mansuy, an elderly, self-made palaeontologist who was the first to describe the now famous Cambrian fossil sites of Yunnan. In 1914, however, he provided facilities to Madeleine Colani, a palaeobotanist and prehistorian, whom he trained and encouraged but who later became one of his fiercest critics.

Between 1909 and 1914, Deprat's boss was Honoré Lantenois, chief mining engineer for Indochina, a strange character who, although officially praising Deprat's skill, was jealous of his fame and disapproved of his lack of respect for his seniors. Lantenois left Indochina in 1914 on war service, but returned to Hanoi in February

1917. A month later, the Deprat case arose.

Deprat was at the peak of his fame — national and international — when Lantenois called him into his office and, apparently embarrassed, told him that Mansuy had accused him (Deprat) of "salting" his fossil collections from Yunnan, Tonkin and northern Annam with European trilobites. Deprat was staggered, as Mansuy had never alluded to the deception. Two weeks later, Deprat returned to one of the suspected locations (Nui Nga Ma, now Nui Nguu Ma, an easily accessible hill near Ben Thuy, along the Hanoi–Saigon highway) and collected another fragment of the trilobite species he had recorded there in 1912. But this was apparently not enough to convince Lantenois.

Lantenois secretly sent the suspected samples to authorities in Paris (Lacroix, Douvillé and Termier) who concluded that the specimens were identical to species from Europe (Wales and Bohemia). In 1918, a committee of enquiry was sent to Nui Nga Ma but failed to find any fossils. There then followed a long and complex trial involving the Academy of Sciences and the Geological Society of France, but Deprat's undiplomatic behaviour steadily worsened his case and Lantenois' political influence and hatred of Deprat was decisive. Deprat was dismissed in November 1920.

Deprat would have been quickly forgotten by academics had it not been for the publication of *Les chiens aboient*. This novelistic account of his case seems accurate, yet the story of his discovery of an additional Ordovician trilobite in Nui Nga Ma in March 1917 remains contentious: neither I nor any Vietnamese palaeontologist has ever found fossils on this hill, a thick Mesozoic conglomerate overlying Ordovician quartzites.

The book caused turmoil in French geological circles, as several of the pseudonyms used were obvious; a complete 'key' was recently given to Durand-Delga by Deprat's daughter, revealing many other famous characters of French geology and palaeontology. Nevertheless, *Les Chiens aboient* was not a bestseller: only 2,500 copies had been sold by 1932 — and backbiters said that many had been purchased by Lantenois himself.

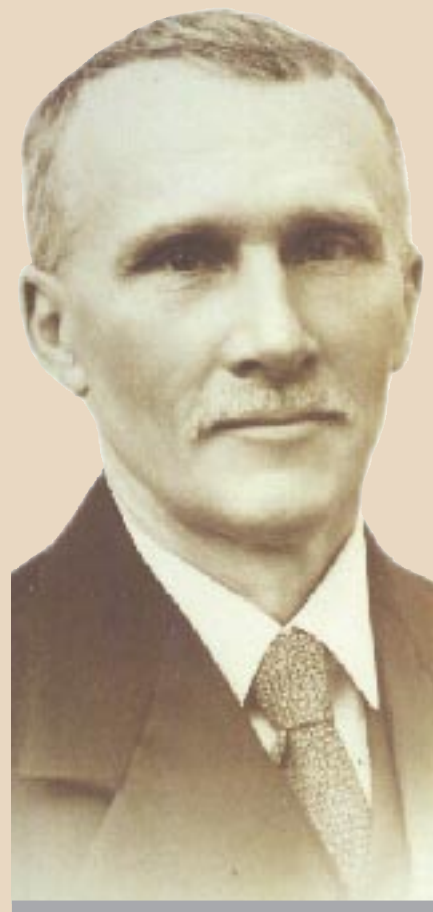
Although most of Deprat's geological work on Indochina was later authenticated by Jacques Fromaget, a famous specialist of this region, the mystery of the trilobites remains. The 10 specimens on which the Deprat affair rests were long considered lost but one of them has recently been rediscovered in a drawer at the Collège de France by Jacques Sigal. It is now in the Muséum National d'Histoire Naturelle in Paris.

Whatever sympathy one may have for Deprat, one must assume that the specimens are indeed apocryphal unless proved otherwise by new field investigations. How they reached Vietnam is unknown. Some accused Mansuy, who revisited France several times (unlike

Deprat, who did not return until 1919). Had Deprat perpetrated the fraud, why did his samples include only a few trilobites among the thousands of other fossils he carefully collected for stratigraphy? And why would he have ruined a promising career with such a useless deception? It seems entirely at odds with his personality and the integrity of his other work.

All the protagonists of the Deprat case are now dead. Deprat died in a climbing accident in the Pyrenees in 1935; ironically, his last novel, *La paroi de glace* ("The ice wall"), published posthumously in 1937, is a detective story dealing with just such an accident. The clue to the case may one day turn up in a document hidden in a drawer somewhere in Vietnam or Paris. Until then, we eagerly await an English translation of *Les chiens aboient* annotated by a historian of geology.

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A largely autobiographic novel about a case of palaeontological fraud – so far unresolved – which shattered French geology

An intracellular protein that binds amyloid- β peptide and mediates neurotoxicity in Alzheimer's disease

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Amyloid- β is a neurotoxic peptide which is implicated in the pathogenesis of Alzheimer's disease. It binds an intracellular polypeptide known as ERAB, thought to be a hydroxysteroid dehydrogenase enzyme, which is expressed in normal tissues, but is overexpressed in neurons affected in Alzheimer's disease. ERAB immunoprecipitates with amyloid- β , and when cell cultures are exposed to amyloid- β , ERAB inside the cell is rapidly redistributed to the plasma membrane. The toxic effect of amyloid- β on these cells is prevented by blocking ERAB and is enhanced by overexpression of ERAB. By interacting with intracellular amyloid- β , ERAB may therefore contribute to the neuronal dysfunction associated with Alzheimer's disease.

Some of the proteolytic fragments of amyloid- β precursor protein (APP) are cytotoxic, causing neuronal dysfunction and death in Alzheimer's disease^{1–3}. Attention has focused on the mechanisms by which extracellular, plaque-associated amyloid- β peptide (A β) exerts its effects on cells. Aggregates of A β comprising residues 1–40/42, especially those that assemble into fibrils and are present in large amounts late on in the disease, are cytotoxic because they disturb the integrity of cell membranes and stimulate the production of reactive oxygen intermediates⁶, eventually causing cell death^{7–9}. But, more important, early in the course of Alzheimer's, before abundant large fibrils impinge on the cells, stronger and more specific interactions with cell-surface receptors for A β ^{10–12} may trigger signal transduction. The receptor RAGE, of the immunoglobulin superfamily, is one such docking site on the neuronal surface that binds A β , thus promoting stress by cellular oxidants¹⁰.

Another important mechanism of cytotoxicity of A β species could result from their engagement by intracellular targets. Intracellular accumulation of amyloidogenic A β occurs in several cell types^{13–19}; this could reflect cellular processing with excessive amounts of A β (of relative molecular mass 4,000; M_r 4K) generated within the cell, or from uptake of released A β . We therefore considered that A β and A β -containing polypeptides might act at specific intracellular sites to augment their neurotoxicity. Using the yeast two-hybrid system²⁰, we have identified an intracellular A β -binding protein which mediates the cellular toxicity of A β . We provide direct evidence for an intracellular binding site for A β and propose that intracellular A β , in addition to A β in the extracellular space, exerts deleterious effects on cellular functions.

Identification and cloning of ERAB

The yeast two-hybrid system was used to screen human brain and HeLa cell complementary DNA libraries for encoded proteins capable of binding to a fusion protein containing amyloid β -

peptide. To screen the libraries, the portion of human amyloid precursor protein sequence encoding A β was subcloned into the yeast expression vector pGBT9 (Fig. 1a), and expression of the A β epitope was confirmed by immunoblotting. Out of 3×10^6 clones screened from each library, one positive clone was identified from human brain and three from HeLa. All four clones had the same cDNA sequence, which we termed ERAB, apart from minor variations at the 5'-end cloning site (pAB002 shown in Fig. 1b, c is a representative ERAB-containing clone). β -Galactosidase activity was strong when the plasmid encoding A β (1–42 or 3–42) and plasmid encoding ERAB were co-expressed (Fig. 1b, c). The specificity of ERAB interaction with A β was confirmed by the lack of β -galactosidase activity when the A β -related construct or the ERAB construct was replaced with vector-only constructs (Fig. 1b) or by constructs encoding three irrelevant proteins (Fig. 1c). The construct pGBT/APP23, which contained amino acids derived from β -amyloid precursor protein (residues 655–751), had no β -galactosidase activity, indicating that the C terminus of APP prevents A β interaction with ERAB and suggesting that normally expressed APP is not a ligand for ERAB. Using 5' rapid amplification of cDNA ends (RACE), the 5' end of ERAB was cloned; the sequence was found to extend about 18 base pairs (bp) upstream of the first AUG and the start codon of the open reading frame was consistent with Kozak consensus sequences²¹. The full-length cDNA sequence encoded a predicted 262-amino-acid polypeptide (U96132). Comparison with the Swiss-Prot and Protein Data Bank using the FASTA algorithm^{22,23} indicated that the amino-acid sequence of ERAB most closely resembles the family of short-chain alcohol dehydrogenases, including hydroxysteroid dehydrogenases, Ke6, and acetoacetyl-CoA reductases^{24,25}. In both cases, ERAB had an identity score of 32% and a similarity score of 65%. Alignment of ERAB with the 255 amino acids of the NADH-binding enzyme 3 α ,20 β -hydroxysteroid dehydrogenase (EC1.1.1.53) of *Streptomyces hydrogenans*²⁵ revealed a series of common structural motifs (Fig. 1d); both share the

NAD(H) binding domains, as well as the highly conserved sequence YGASK in the bacterial enzyme (residues 153–157), which in human ERAB corresponds to residues 169–173 (YSASK), putatively assigned as part of the active centre of the enzyme.

To study binding of ERAB to A β , we expressed a His-ERAB fusion protein in *E. coli* and purified it. Immunoblotting with polyclonal antibodies prepared by immunizing with ERAB-derived peptides (anti-ERAB IgG) indicated the presence of the target ERAB sequence in the fusion protein, but not in the control fusion protein, His-tagged chloramphenicol acetyltransferase (His-CAT) (Fig. 2a, lanes 1 and 2). The specificity of anti-ERAB IgG was demonstrated by its recognition of the His-ERAB fusion protein in crude cell lysates, and by the disappearance of the immunoreactive band on addition of excess free ERAB-derived peptides identical to that used as immunogen (Fig. 2a, lane 3). For analysis of the interaction between ERAB and A β , we used non-radioactive and radioactive ligand-binding assays: 125 I-labelled His-ERAB and A β (1–42) adsorbed onto microtitre wells bound specifically according to the concentration of A β (Fig. 2b) and was blocked in a dose-dependent manner by increasing amounts of unlabelled His-ERAB (Fig. 2c), but not by His-CAT. The specificity of 125 I-His-ERAB binding to immobilized A β was confirmed by studies with antibodies raised

against ERAB; anti-ERAB IgG blocked binding of 125 I-His-ERAB to A β , whereas nonimmune IgG had no effect (Fig. 2d). Experiments in which a single concentration of A β (1–42) was immobilized on wells and varying amounts of 125 I-His-ERAB were added showed dose-dependent binding, with a dissociation constant (K_d) of 88.3 ± 28.1 nM (Fig. 2e). To rule out the possibility of artefacts due to radioiodination, binding experiments in which A β was immobilized on wells followed by addition of ERAB were repeated using antibody to detect bound ERAB: K_d was 42 ± 8 nM (Fig. 2f), similar to the results obtained with 125 I-ERAB. Studies with A β -related peptides demonstrated that A β (1–40) and A β (1–20), but not A β (25–35), bound to ERAB with comparable affinity, although unrelated peptides, including amyloidogenic amylin and prion fragment 109–141, showed no significant affinity for ERAB (C. Soto and S.-D. Yan, unpublished data). Monitoring fluorescence arising from ERAB–A β interaction gave similar binding parameters.

Tissue and cellular localization of ERAB

ERAB messenger RNA is expressed ubiquitously in normal human tissues as a single transcript of 1 kilobase (kb) (Fig 3a–c); levels of ERAB transcripts were highest in liver and heart, but ERAB was also

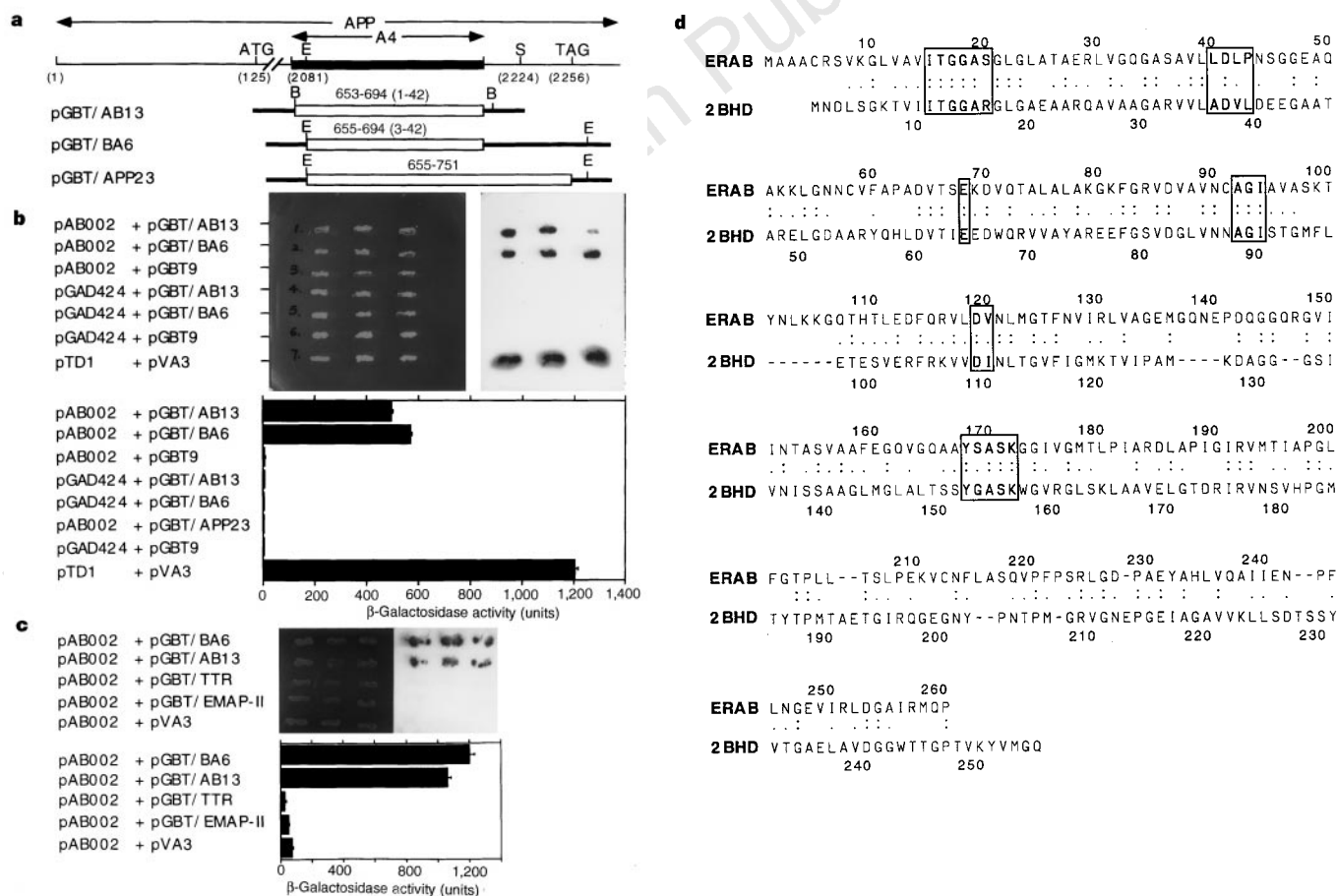


Figure 1 Identification and cloning of a cell-associated binding protein for A β . **a**, Fragments of human APP cloned into the yeast expression vector pGBT9. The line at the top shows full-length APP cDNA (numbered according to the Genebank sequence X06989); the thickened portion indicates the region encoding A4/A β (APP amino acids 653 to 694). ATG, start codon; TAG, stop codon; E, *Eco*RI; S, *S*tyl; and B, *B*amHI. **b**, Expression of β -galactosidase activity when yeast were co-transformed with the two indicated constructs. Left, yeast is present on the agar; right, same area showing the β -galactosidase reaction product; the lower panel shows quantification of β -galactosidase activity. pAB002 is one of three ERAB-containing clones derived from the HeLa cell Matchmaker

cDNA library. Co-transformation with pTD1 (SV40 large T-antigen) and pVA3 (murine p53) is the positive control. **c**, Specificity of the ERAB-A β interaction in the yeast two-hybrid system. The indicated unrelated protein (transferrin (TTR); K02091, murine p53, or endothelial-monocyte activating polypeptide II (EMAPII); U10117) was subcloned into pGBT9. **d**, Alignment of the ERAB deduced amino-acid sequence with that for 20- β -hydroxysteroid dehydrogenase (2BHD); boxed amino-acid domains are likely to be involved in binding of nicotinamide adenine dinucleotide (NAD). The box containing residues 169–173 of ERAB corresponds to the putative active centre of the steroid-binding domain. One dot and two dots indicate similar and identical amino-acid residues, respectively.

expressed in normal brain. ERAB antigen, detected with polyclonal anti-ERAB peptide-derived antibody, was visualized as a 27K band in extracts of normal brain (Fig. 3d, lanes 5–7). This apparent relative molecular mass from SDS–PAGE is close to that predicted from the ERAB sequence (M_r 26,925.7). The intensity of the band on immunoblots of material derived from the brains of four different patients with Alzheimer's (Fig. 3d, lanes 1–4) was greater than that derived from brain extracts from age-matched controls (Fig. 3d, lanes 5–7). Immunostaining of normal brain showed that ERAB was present at low levels and was predominantly localized in neurons (Fig. 3e); neuronal ERAB expression was increased in Alzheimer's brain (Fig. 3f), especially near deposits of A β (Fig. 3f, inset).

Histological evidence and the apparent absence of a signal

peptide or transmembrane-spanning domain indicated that ERAB was intracellular. Immunoblotting demonstrated that ERAB was expressed in both human neuroblastoma (SK-N-SH) and HeLa cells as a single 27K band whose appearance was blocked by excess free His-ERAB. Confocal microscopy of HeLa cell cultures showed that ERAB was distributed (Fig. 4a) with the endoplasmic reticulum marker protein disulphide isomerase (PDI) (Fig. 4b), as depicted in double immunofluorescence images for both antigens (Fig. 4c). In neuroblastoma cells transfected to overexpress ERAB, subcellular fractionation showed ERAB antigen in fractions containing the endoplasmic reticulum marker GRP78 (Fig. 4d, lanes 4–5), but not in fractions containing plasma membrane, cytosol or Golgi (Fig. 4d, lanes 1–3). Consistent with this intracellular localization, ERAB was not detectable on the cell surface when labelled anti-ERAB IgG was

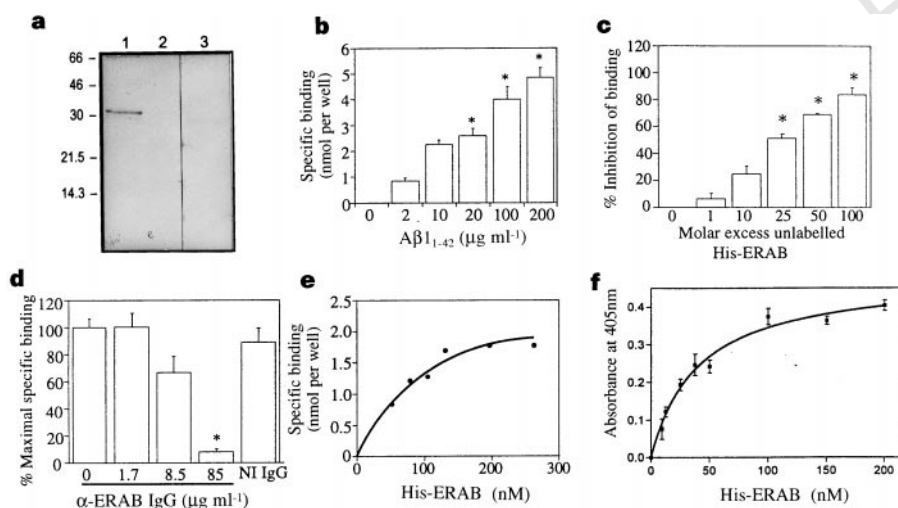
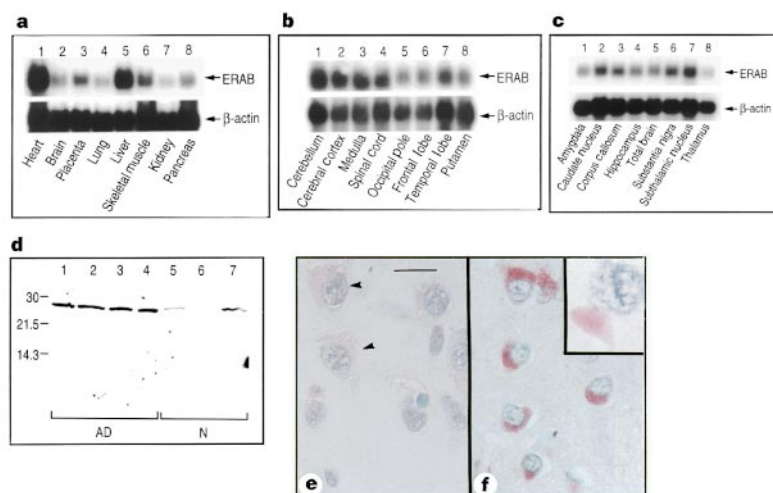


Figure 2 Expression of ERAB fusion protein: A β -binding properties. **a**, SDS–PAGE (12%; reduced) followed by immunoblotting of His-ERAB (lane 1; 2 μ g) and His-CAT (lane 2; 2 μ g) fusion proteins using anti-ERAB IgG (3 μ g ml⁻¹). Lane 3 carries the same sample as lane 1, except that free peptide used as immunogen was present at 25-fold molar excess over the antibody. **b–e**, Specific binding of ¹²⁵I-His-ERAB to A β (1–42) adsorbed to microtitre wells. In **b**, the indicated concentration of A β (1–42) was incubated in wells, excess sites on the plate were blocked, and binding was assayed by adding ¹²⁵I-His-ERAB (50 nM) alone or in the presence of an 100-fold molar excess of unlabelled His-ERAB. In **c**, A β (1–42) (5 μ g) was incubated in wells, and binding was assayed with ¹²⁵I-His-ERAB (50 nM) in the presence of increasing amounts of unlabelled His-ERAB. In **d**, A β (1–42) (5 μ g) was incubated in wells and binding assay with ¹²⁵I-His-ERAB

(50 nM) alone or in the presence of a 100-fold excess His-ERAB; as indicated, either anti-ERAB (α -ERAB) or non-immune (NI) IgG (85 μ g ml⁻¹) was added. In **e**, binding was assayed in the presence of the indicated concentration of ¹²⁵I-His-ERAB (alone or with a 100-fold molar excess of unlabelled His-ERAB) in wells with adsorbed A β (1–42) (5 μ g per well). Zero indicates wells coated with albumin alone. In each case (**b–e**), the mean $\pm$ s.e.m. is shown of specific binding (binding in wells incubated with ¹²⁵I-His-ERAB alone (total binding) minus binding in wells incubated with ¹²⁵I-His-ERAB in the presence of a 100-fold molar excess of unlabelled His-ERAB (nonspecific binding)) of quadruplicates. **f**, Wells were coated with A β (1–42), excess sites were blocked with albumin (1%), and the indicated amounts of ERAB added. Bound ERAB was quantified by ELISA using anti-ERAB IgG as primary antibody. Asterisk indicates $P < 0.01$.

Figure 3 Expression of ERAB in human tissue. **a–c**, Northern analysis of total RNA from normal organs (**a**) or brain subregions (**b**, **c**) hybridized with full-length ³²P-labelled human cDNA for ERAB. Controls were the same blots hybridized with ³²P-labelled cDNA encoding β -actin. **d**, Immunoblot of brain extracts with anti-ERAB IgG. In lanes 1–4 (designated AD) and 5–7 (designated N), extracts were prepared from temporal lobes of four different AD and three age-matched normal (control) brains, respectively. **e**, **f**, Immunostaining for ERAB in age-matched normal (**e**) and AD (**f**) brain. Arrows in **e** point to representative neurons. The inset in **f** shows double-stained A β (grey; murine monoclonal anti-A β IgG)¹⁰ and ERAB (pink). Scale bar in **e** and **f** is 29 μ m.



used. Electron microscopy confirmed the presence of ERAB in the endoplasmic reticulum and also showed antigen associated with mitochondria (S.-D. Yan, unpublished result).

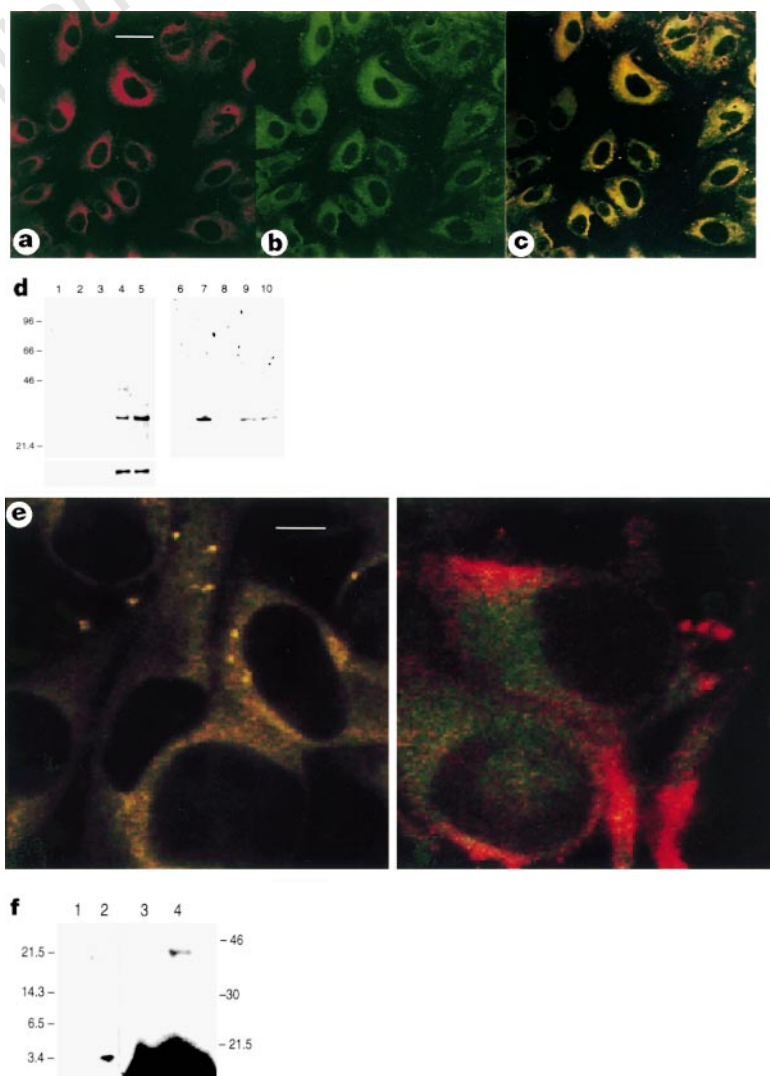
As ERAB was mainly present in microsomes, we investigated how it might interact with A β . First, we found that A β influenced the distribution of ERAB: addition of A β (1–42) to neuroblastoma cells markedly changed the cellular localization of ERAB from its original distribution which overlapped with PDI (Fig. 4e, left panel), resulting in the accumulation of ERAB antigen (red fluorescence) at the inner side of the cell membrane away from PDI (green fluorescence) (Fig. 4e, right panel); subcellular fractionation of neuroblastoma cells also showed that preincubation with A β shifted most ERAB antigen to the plasma membrane fraction (Fig. 4d, lane 7). Second, we found that ERAB became associated with A β , and that A β induced effects on ERAB that were not only indirect. When SK-N-SH cells were incubated with 125 I-A β (1–40) and cell-associated peptide was immunoprecipitated using antibodies against ERAB, anti-ERAB IgG precipitated a 125 I-labelled 4K band on Tris-tricine gels corresponding to A β (Fig. 4f, lane 2), whereas non-immune IgG did not precipitate any bands (Fig. 4f, lane 1). To confirm this interaction of ERAB and A β in intact cultured cells, we incubated neuroblastoma cells at 37°C with 125 I-A β , added the crosslinker disuccinimidyl suberate, and immunoprecipitated the reaction. Antibody to ERAB precipitated a 125 I-labelled band of M_r 38K (Fig. 4f, lane 4) which was probably a complex of ERAB with a dimer of 125 I-A β , but non-immune IgG gave no precipitate (Fig. 4f,

lane 3). These results show that A β affects the cellular distribution of ERAB and that ERAB–A β interaction occurs in intact cells.

ERAB and A β -mediated cellular toxicity

We investigated the role of ERAB in A β -induced cellular stress in order to assess its functional significance. First, we evaluated the effect of ERAB expression on the sensitivity of neuroblastoma cells (which normally express ERAB) to A β -induced stress using blocking antibody fragment F(ab')₂ prepared from affinity-purified anti-ERAB IgG. Two criteria were used to study A β -induced cellular perturbation: suppression of the cellular capacity to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)²⁶, and induction of apoptosis⁹. Anti-ERAB or non-immune F(ab')₂ was introduced into neuroblastoma cells using liposomes; after introduction of the F(ab')₂, SK-N-SH cultures were incubated with a range of A β (1–42) concentrations. Whereas cultures exposed to non-immune-derived F(ab')₂ or liposomes alone showed marked A β -induced suppression of MTT reduction, liposome/anti-ERAB F(ab')₂ protected, in part, against A β -induced suppression of MTT reduction (Fig. 5a). In contrast, addition of anti-ERAB F(ab')₂ directly to the medium (not in liposomes) had no effect on A β -induced MTT suppression, consistent with the intracellular localization of ERAB (S.-D.Y., unpublished observation). Apoptosis, another indicator of A β -induced cytotoxicity, was monitored by enzyme-linked immunoabsorbent assay (ELISA) for DNA fragmentation, and by the terminal deoxynucleotidyl transferase-mediated

Figure 4 Expression of ERAB in cultured cells: localization to the endoplasmic reticulum and change in distribution following addition of A β . **a–c**, Confocal microscopy demonstrating immunofluorescence staining for ERAB alone (**a**; red), protein disulphide isomerase (PDI) alone (**b**; green), or these two antigens simultaneously co-localized (**c**; yellow). Scale bar in **a–c** is 25 μ m. **d**, Transfected cells (5×10^5) were pelleted and fractionated in a series of sucrose steps (38, 30 and 20%) by ultracentrifugation: layered (lanes 1–4) and pelleted fractions (lane 5) were western blotted using either anti-ERAB IgG (top) or anti-GRP78 IgG (bottom). Lanes correspond to cytosol (lanes 1 and 6), plasma membrane (lanes 2 and 7), Golgi apparatus (lanes 3 and 8), and endoplasmic reticulum (lanes 4, 5 and 9, 10). In lanes 6–10, subcellular fractionation of ERAB-transfected human neuroblastoma cells was performed following exposure to A β (1–42) (1 μ M) for 14 h at 37°C. **e**, Neuroblastoma cells were incubated in buffer alone or in the presence of A β (1–42) (1 μ M) for 14 h at 37°C, and the distribution of ERAB determined by double-immunofluorescence staining for ERAB (red) and PDI (green). The two antigens, which were initially co-localized (yellow, left), then separate, allowing visualization of ERAB deposits near the cell membrane (red) distinct from PDI (green) (right). Scale bars, 6 μ m. **f**, Neuroblastoma cells (2×10^7) were incubated with 125 I-A β (100 nM) for 6 h at 37°C, and cells dissolved in lysis buffer¹⁰ followed by immunoprecipitation with anti-ERAB IgG (10 μ g ml^{–1}; lane 2) or non-immune IgG (10 μ g ml^{–1}; lane 1), and Tris-tricine gel electrophoresis. Alternatively, neuroblastoma cells (2×10^7) were incubated with 125 I-A β (100 nM) for 6 h at 37°C, disuccinimidyl suberate (0.2 mM; Pierce) was added for 30 min at 25°C, cells were washed extensively, followed by dissolution of cells in lysis buffer and electrophoresis on non-reducing SDS-PAGE (10%); immunoprecipitation with non-immune IgG (lane 3) and anti-ERAB IgG (lane 4).



dUTP nick-end labelling (TUNEL) assay. Cultures exposed to liposome/anti-ERAB $F(ab')_2$ showed inhibition of $A\beta$ -induced apoptosis, compared with cultures pretreated with liposome/non-immune $F(ab')_2$ or liposomes alone (Fig. 5b). To correlate induction of apoptosis and cell uptake of $F(ab')_2$, a relative apoptosis index (RAI) was used, representing the ratio of apoptotic nuclei (by TUNEL) in cells staining positively for $F(ab')_2$ divided by the total number of cells that took up $F(ab')_2$ in the same cell population (Fig. 5c). Cells into which anti-ERAB $F(ab')_2$ had been introduced (Fig. 5d, top) showed diminished apoptosis ($RAI_{\alpha ERAB} = 10\text{--}15\%$; Fig. 5c, d, bottom) after addition of $A\beta$, compared with cells in the same well that did not take up the $F(ab')_2$ ($RAI_0 = 60\text{--}70\%$). In contrast, cells exposed to liposome/non-immune $F(ab')_2$ (Fig. 5e, top) had 60–70% apoptotic nuclei (Fig. 5c, e bottom) after addition of $A\beta$, whether the cells took up non-immune $F(ab')_2$ or not (RAI_{NI} or RAI_0 , respectively).

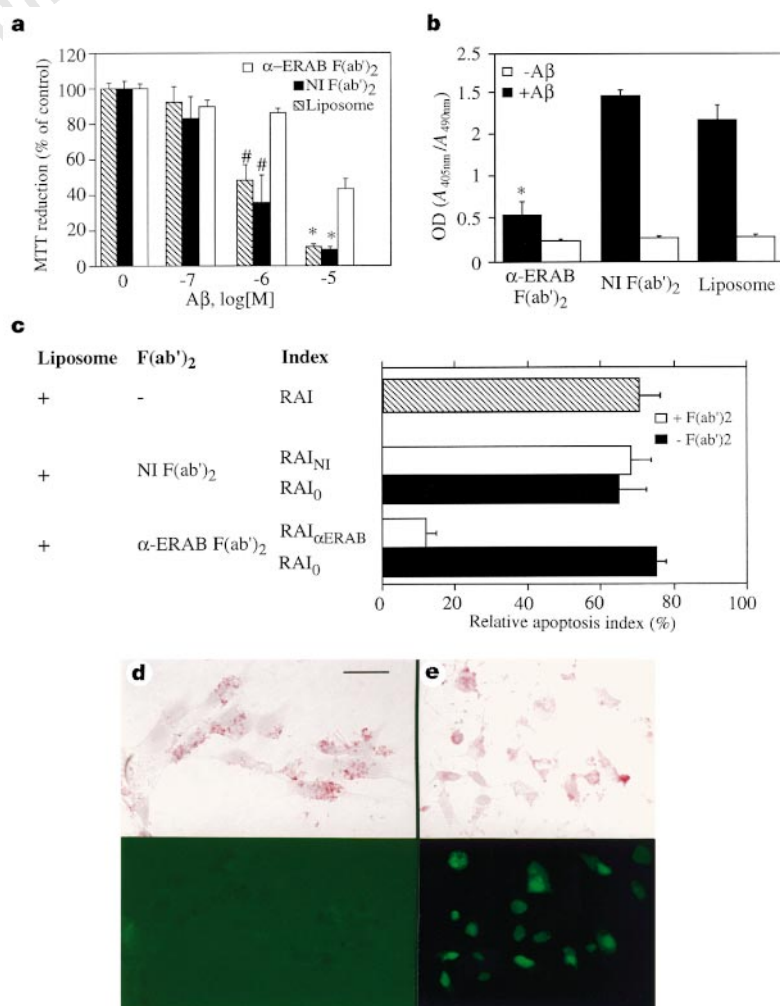
We also used transfection to boost the expression of ERAB in order to evaluate its effect on $A\beta$ -induced stress and cytotoxicity. COS cells, which express very low levels of endogenous ERAB, were transfected with plasmids encoding ERAB (pcDNA3/ERAB) and/or $A\beta$ (pcDNA3/ $A\beta$), resulting in their overexpression compared with mock-transfected controls. First, when cultures transfected with pcDNA3/ERAB alone were exposed to exogenous $A\beta$, their capacity to reduce MTT was suppressed compared with mock-transfected controls (Fig. 6a). Second, to assess the effect of high levels of endogenous ERAB and $A\beta$ on apoptosis, COS-1 cells were cotransfected with pcDNA3/ERAB and pcDNA3/ $A\beta$; this caused a prominent rise in apoptosis compared with mock-transfected cultures

(pcDNA3 alone) or those transfected with either pcDNA3/ $A\beta$ or pcDNA3/ERAB (Fig. 6b). To correlate expression of ERAB and/or $A\beta$ in cells with the occurrence of apoptosis, the ratio of these two parameters, a modified RAI, was developed by determining the percentage of apoptotic nuclei (using TUNEL assay) in cells staining positively for ERAB and/or $A\beta$ in the same cell population (Fig. 6c). Co-transfected cells that overexpressed ERAB and $A\beta$ constituted a population in which 65% of the nuclei stained positively TUNEL assay (defined as $RAI_{ERAB+A\beta}$). In contrast, cells in the same wells not successfully transfected (that is, they did not express ERAB or $A\beta$) showed only 5–10% positive nuclei by TUNEL assay (defined as RAI_0 ; Fig. 6c). Transfection of COS-1 cells with either pcDNA3/ERAB or pc-DNA3/ $A\beta$ alone showed only a small increase in apoptosis in cells expressing either of these, compared with non-transfected cells in the same dish; this value was only slightly greater than that in mock-transfected cultures (5–10% apoptosis; Fig. 6c).

Discussion

Neurotoxicity in Alzheimer's disease may result from a concatenation of factors such as enhanced cellular processing of APP, releasing amyloidogenic $A\beta$ peptides; interaction of $A\beta$ with cell membranes, both at specific cell-binding sites and, presumably, by directing action on the membrane itself; non-enzymatic glycation of macromolecules whose turnover is delayed in Alzheimer's diseased brain, producing modified structures that can form crosslinks and generate reactive oxygen intermediates; and abnormality in the protective response to oxidant stress and/or susceptibility to apoptotic stimuli^{1–12,26–30}. Mutant presenilins, associated with most familial

Figure 5 ERAB contributes to $A\beta$ -induced cytotoxicity in neuroblastoma cells. **a**, Effect of anti-ERAB $F(ab')_2$ on $A\beta$ -induced suppression of MTT reduction. SK-N-SH cells were incubated with liposome/anti-ERAB or non-immune $F(ab')_2$ (18 μ g $F(ab')_2$), or liposomes alone in order to introduce proteins into the cell⁵⁰, and, 24 h later, cultures were incubated with the indicated concentrations of $A\beta$ (1–42) for 20 h at 37°C. The mean $\pm$ s.e.m. of six replicates is shown. Asterisks and hash symbols $P < 0.01$ and $P < 0.05$, respectively. **b–e**, Effect of anti-ERAB $F(ab')_2$ on $A\beta$ -induced apoptosis. **b**, ELISA for DNA fragmentation. Anti-ERAB $F(ab')_2$ or non-immune $F(ab')_2$ was introduced into neuroblastoma cells as described for the first 24 h; cultures were then incubated with $A\beta$ (1–42) (2.5 μ M) for further 24 h. Apoptosis was determined using a cell-death detection assay. **c**, Relative apoptosis index (RAI) correlating $A\beta$ -induced apoptosis and the protective effect of anti-ERAB $F(ab')_2$ versus non-immune $F(ab')_2$. SK-N-SH cells were preincubated with liposomes alone, liposome/anti-ERAB $F(ab')_2$ or liposome/non-immune $F(ab')_2$ and $A\beta$ as described. At 48-h, apoptosis was determined by TUNEL staining and cell uptake of $F(ab')_2$ was determined immunohistochemically. $RAI_{\alpha ERAB}$ and RAI_{NI} are percentages denoting the number of apoptotic nuclei from cells that took up the indicated $F(ab')_2$ divided by the total number of cells that took up either anti-ERAB $F(ab')_2$ or non-immune $F(ab')_2$, respectively. RAI_0 denotes the percentage of apoptotic nuclei from cells that did not take up $F(ab')_2$, divided by the total number of cells in the same cell population that did not take up $F(ab')_2$ in each of the experiments. RAIs were determined by counting positive cells, mean $\pm$ s.e.m., of ten high-power fields. Asterisk, $P < 0.01$. **d**, **e**, Representative micrographs of SK-N-SH cells into which anti-ERAB $F(ab')_2$ was introduced (**d**, upper), and TUNEL assay was performed (**d**, lower) on the same cells and cells into which non-immune $F(ab')_2$ was introduced (**e**, upper) and the TUNEL assay was performed (**e**, lower). Scale bar, 20 μ m.



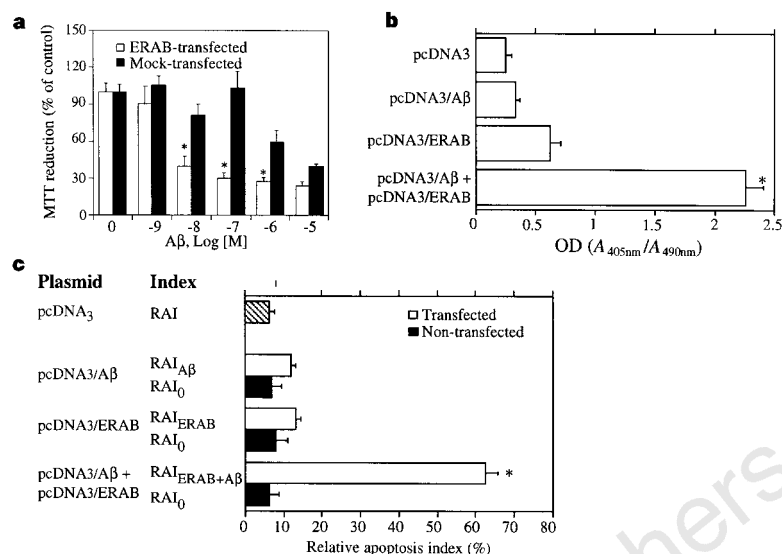


Figure 6 ERAB contributes to A β -induced cellular toxicity in ERAB-transfected COS cells. **a**, ERAB-transfected COS cells enhance suppression of MTT reduction in the presence of A β . COS cells were transiently transfected with the pcDNA3/ERAB or pcDNA3 alone, and 24 h later (at which time ERAB was expressed at high levels) A β was added for 24 h at 37°C, when MTT reduction was determined²⁶; mean $\pm$ s.e.m. of six replicated determinations is shown. Asterisk, $P < 0.05$. **b**, **c**, Overexpression of ERAB and/or A β in COS cells: effect on apoptosis. **b**, ELISA for DNA fragmentation was studied on COS cells co-transfected with pcDNA3/ERAB + pcDNA3/A β , or transfected with either pcDNA3/ERAB, pcDNA3/A β or pcDNA3 alone. **c**, RAI correlating A β -mediated apoptosis and overexpression of ERAB and/or A β in COS cells. COS cells were induced to overexpress ERAB and A β (by transfection with pcDNA3/ERAB +

pcDNA3/A β), ERAB alone (pcDNA3/ERAB) or A β alone (pcDNA3/A β). At 72-h, apoptosis was determined by TUNEL staining and expression of ERAB and/or A β was analysed immunocytochemically: RAI_{ERAB+A β} , RAI_{ERAB} and RAI_{A β} are percentages denoting the number of apoptotic nuclei from cells expressing either ERAB and A β (following co-transfection of pcDNA3/ERAB + pcDNA3/A β), ERAB alone (following transfection of pcDNA3/ERAB), or A β alone (following transfection of pcDNA3/A β) divided by the total number of cells expressing that antigen. RAI₀ is a percentage denoting the number of apoptotic nuclei from cells not expressing any of the transfected gene(s) in the same cell population from each of three different experiments, divided by the total number of cells that did not express the indicated gene product. RAIs were determined by counting positive cells, mean $\pm$ s.e.m., in ten high-power fields. In **b** and **c**, an asterisk denotes $P < 0.01$.

forms of Alzheimer's disease^{31–33} and present in the endoplasmic reticulum and Golgi complex³⁴, provide an example of this multifactorial pathogenesis. Increased production of A β occurs in transgenic mice overexpressing mutant presenilins, and in the plasma and cultured fibroblasts from carriers of familial Alzheimer's disease linked to chromosome 1 (refs 35–37). In addition, presenilin 2, which is homologous to ALG-3, a protein involved in programmed cell death, can function as a susceptibility factor for apoptosis in differentiated PC12 cells, in response to growth factor/serum withdrawal or to addition of A β ³⁸.

ERAB adds a new dimension to the pathogenetic interaction of A β with cellular elements. Like mutant presenilin 2 (ref. 38), overexpressed ERAB also appears to potentiate cellular perturbation due to A β . However, the physiological roles of ERAB and the presenilins remain mostly unknown, although it has been found that presenilins participate in neural development^{39,40}. Addition of A β to COS cells overexpressing ERAB caused more suppression of MTT reduction and enhanced apoptosis compared with COS cells not overexpressing ERAB. Similarly, in neuroblastoma cells, which constitutively express ERAB, A β -induced impairment of MTT reduction and A β -mediated apoptosis were suppressed by the blocking anti-ERAB F(ab')₂. We therefore proposed that ERAB may contribute to the pathogenesis of Alzheimer's by being an intracellular target for A β , mediating cellular stress and, ultimately, apoptosis due to increased amounts of A β . Although the mechanism by which ERAB promotes A β -mediated changes in cellular function is not clear, ERAB and A β appear to interact in intact cells.

The ERAB sequence indicates that ERAB might be an enzyme such as a hydroxysteroid dehydrogenase or an acetoacetyl-CoA reductase. The interaction of A β with such an intracellular polypeptide is surprising, and the effect of A β on the putative enzymatic activity of ERAB needs investigation. But how can A β gain access to

ERAB that is apparently confined to the intracellular compartment? A β , A β aggregates and amyloid fibrils have been found inside cells^{13–16,41–45} and A β can be generated in the endoplasmic reticulum^{13,16} and taken up by endocytosis^{41,44–46}, suggesting that some A β can get into the cell.

Although ERAB transcripts are widely distributed in the body, with expression being highest in liver and heart, we do not know how ERAB participates in cellular homeostasis. By virtue of its association with the endoplasmic reticulum and mitochondria, and its resemblance to alcohol dehydrogenases, we propose that ERAB normally functions in cellular metabolism and biosynthesis, and that such functions may be perturbed by A β . We are now investigating how ERAB affects the function of normal and perturbed cells. *Note added in proof:* A human gene identical to ERAB and mapped to the X chromosome has been entered in GenBank by O. P. Zuchenko *et al.* (unpublished); also, S. Furuta *et al.* have reported the cloning of a bovine cDNA that is the counterpart of human ERAB (*Biochim. Biophys. Acta* **1350**, 317–324; 1997). □

Methods

Yeast two-hybrid system. Fragments of human amyloid precursor protein (APP) were cloned into the yeast expression vector pGBT9 (Clontech). The 3' end of the *EcoRI* fragment from the APP cDNA was cloned into pGBT9 to generate pGBT/APP23, which encodes a fusion protein comprising the C terminus of APP (amino acids 655–751). A stop codon (TGA) was introduced after the sequence of A4/A β by site-directed mutagenesis and the resulting *EcoRI* fragment was cloned into pGBT9 to generate pGBT/BA6, which encodes a fusion protein containing the A4/A β peptide sequence (amino acids 3–42). A *BamHI* site was introduced at the beginning of A4/A β by PCR amplification and the resulting fragment was subcloned into the unique *BamHI* site of pGBT9 to generate pGBT/AB13, the latter encoding a fusion protein with the A4/A β peptide sequence (amino acids 1–42). The plasmid constructs pGBT/BA6

and pGBT/AB13 were used as bait to screen human brain and HeLa Matchmaker libraries according to the manufacturer's instructions. pGAD424, pGAD GH and pGAD3F are yeast expression vectors encoding the GAL4 transactivation domain (Clontech). β -Galactosidase activity was assayed using *o*-nitrophenyl β -D-galactopyranoside (ONPG) as substrate.

Ligand binding studies. A fusion-protein construct was prepared by subcloning ~1,116-bp *EcoRI* fragment of ERAB cDNA (including 318 bp of 3' untranslated sequence) into the unique *EcoRI* site of pTrcHis C vector (Invitrogen) to express a His-ERAB fusion protein. Following transformation of *E. coli* with this construct, His-ERAB was purified as described by the manufacturer (Invitrogen). A control His-CAT construct (Invitrogen) was also used to transform *E. coli* and the fusion protein similarly purified. Antibody against ERAB was prepared by immunizing rabbits with ERAB-derived peptides, corresponding to residues 100–116 and 133–147, conjugated to keyhole limpet haemocyanin. These antibodies were specific for ERAB (Fig. 2a and text), and were used for immunoblotting, immunohistochemistry and functional studies. Immunoblotting used anti-ERAB IgG and sites of primary antibody binding were detected using the alkaline phosphatase method (Sigma). The binding assay to demonstrate direct interaction of ERAB and A β utilized a modification of our previously described method¹⁰; ELISA was used for non-radioactive assay⁴⁷.

Immunoblotting and immunocytochemistry. Extracts of human brain temporal lobe were prepared as described¹⁰ and run on SDS-PAGE (12% acrylamide; reducing gels; 100 μ g protein per lane) and immunoblotted. Blots were reacted with rabbit anti-ERAB IgG (3 μ g ml⁻¹) and sites of IgG binding were detected by chemiluminescence (Amersham). For subcellular fractionation, human neuroblastoma cells were transfected with pcDNA3-ERAB using the lipofectamine method¹⁰, and fractionated as described following freeze-thawing, nitrogen cavitation, homogenization and ultracentrifugation⁴⁸. Brains were fixed in paraformaldehyde (4%), paraffin-embedded, sectioned and stained with anti-ERAB IgG (30 μ g ml⁻¹). Non-immune IgG was used as control. Histological results are representative of seven Alzheimer's disease (AD) brains and five age-matched normal brains, less than 8 h post-mortem. For indirect immunofluorescence, cells were plated on coverslips, fixed in formaldehyde (3.7%) for 15 min, and permeabilized in Triton X-100 (0.5%) for 5 min. For co-staining, coverslips were sequentially incubated for 1–1.5 h at room temperature with murine monoclonal anti-protein disulphide isomerase (StressGen), anti-mouse FITC (Pierce), rabbit anti-ERAB IgG and anti-rabbit TRITC Ig G (Pierce).

Transfection and cellular toxicity assays. Transfection of COS cells was achieved with pcDNA3 vectors bearing either the cDNA for ERAB and/or A β using lipofectamine according to the manufacturer's instructions. The AB-containing construct was made as follows: a Kozak consensus sequence (GCC CCA GCC) was added before nucleotide 2,078 of human APP and a stop codon (TAG) was introduced after nucleotide 2206. MTT was reduced and photomicrographs obtained as described^{10,26}. ELISA for DNA fragmentation and TUNEL staining were done according to the manufacturer's instructions (Boehringer Mannheim). A β was detected with rabbit anti-A β (1–42) IgG: this antibody is selective for the C terminus of A β (1–42)⁴⁹. Sites of primary antibody binding were detected with peroxidase-conjugated goat anti-rabbit IgG. Subsequently, ERAB antigen was visualized using fluorescein-conjugated anti-ERAB IgG.

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Detection of Geminga as a radio pulsar

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Geminga was discovered as a strong γ -ray source in the constellation Gemini over two decades ago^{1,2}, and was later detected at X-ray³ and optical⁴ wavelengths. X-ray pulsations⁵ with a period of 237 ms established that it is a rotating neutron star. Although γ -ray pulses were subsequently discovered (once the period was known) in archived data⁶, no evidence for radio emission (either continuum or pulsed) was found; in this respect, Geminga is different from every other neutron star with pulsed emission. Here we report the detection of pulsed 102.5-MHz radio emission from Geminga, with a period of 237 ms. The flux density varies within the range 5–500 mJy and the pulse width varies between 10 and 80 ms. The small dispersion measure ($2.9 \pm 0.5 \text{ pc cm}^{-3}$) confirms Geminga's proximity to the Sun and establishes it as the weakest known radio pulsar. This observation poses a considerable challenge for pulsar emission models, which must now be able to explain the exceptional contrast between the strength of the γ -ray and radio emission from this object.

The search for radio emission from Geminga was started in 1975 by Bignami *et al.*⁷ with a map at 610 MHz centred on the γ -ray position; the search was continued later by many groups at frequencies between 325 MHz and 10.7 GHz using different types of radio telescopes. The observations concentrated either on the continuum^{8–11} or on the pulsed emission^{12–15}, albeit without success. Now, however, there are three groups at the Pushchino Radio Astronomy Observatory (our own, Kuzmin and Losovsky¹⁶, and Shitov and Pugachev¹⁷) reporting independently the detection of pulsed radio emission from Geminga at 102.5 MHz. All groups agree more or less on the value of the dispersion measure, but have obtained different flux density and pulse width values.

Our observations were obtained during 37 days, split into five epochs of four to ten days in December 1996 and January–May 1997, as part of a survey for a weak pulsars at 102.5 MHz. The measurements were carried out with the Large Phased Array (LPA), a sensitive transit antenna consisting of 16,384 dipoles, which has an operating frequency of 102.5 ± 1.5 MHz. The observations were made using a multi-channel receiver, where the individual channels had a width of 20 kHz. This enabled us to remove interstellar dispersion and to suppress interference caused by commercial wireless stations and industrial sources. With this system it is possible to detect pulsars with an average flux as weak as 4 mJy at 102.5 MHz.

During our observations we used a receiver time constant of 3 ms and a sampling interval of 2.56 ms. The first 15 samples of the observing window were occupied by the calibration signal. The exact timing and synchronization with the apparent pulsar period were checked regularly with strong pulsars¹⁸. We precomputed the exact time of arrival (TOA) for Geminga (PSR J0633 + 1746) for each day on the basis of the known period P , derivative $\dot{P}$ and coordinates (J. H. Taylor, R. N. Manchester, A. G. Lyne and F. Camilo, unpublished data; henceforth 'J. H. Taylor *et al.*'). A sum of 954 individual pulses in 32, 64 or 128 independent frequency channels could be recorded at each transit and transferred to disk. We calculated then the standard deviation σ for each channel and deleted those channels which were apparently spoiled by interference. Normally between 15 and 26 channels from every 32 could be used to sum up the mean profile.

We computed the dispersion measure (from the data of the six

best days) from the time delay between the mean profiles obtained by adding the signals in the first six and in the last six channels. We obtained a mean value of $2.9 \pm 0.5 \text{ pc cm}^{-3}$. This is one of the smallest dispersion measures found for all 750 known pulsars (J. H. Taylor *et al.*, unpublished data). The distance to Geminga estimated using a galactic electron density model¹⁹ is $155 \pm 30 \text{ pc}$ and agrees quite well with the parallax-method estimate²⁰ of 157 pc. Based on our dispersion measure and the published distance²⁰ of 157 pc, we obtain the mean electron density $n_e = 0.018 \text{ cm}^{-3}$, in good agreement with the value computed using a simple galactic electron density model to this direction of Galaxy¹⁹. Examples of de-dispersed mean profiles of Geminga are shown in Fig. 1.

The profile shape (Fig. 1) is very similar and narrow on the first two days, and wider on the fourth, when it seems to be more complex on the third day, when, due to interference, we integrated fewer periods. This behaviour, of a two- or three-component profile, was seen during one-third of the 30 days which were used

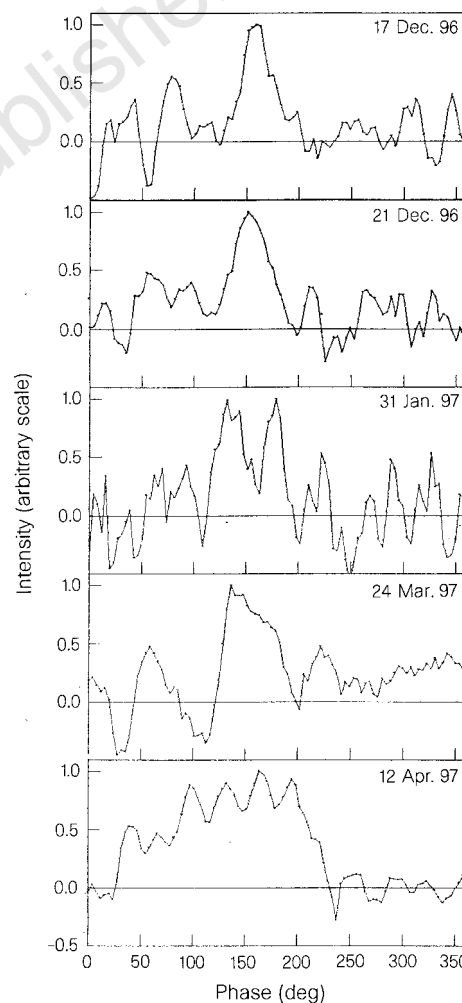


Figure 1 Examples of mean pulse profiles of Geminga for five days (dates shown at top right of each panel). 954 individual pulses have been integrated in four of the days and 318 pulses in one of the days (31 January 1997). Pulse profiles were usually visible at the $(2-4)\sigma$ level in several of the transits in each observing period. To improve the signal-to-noise ratio further, the data were smoothed by use of a running mean over five samples. These profiles had already a signal-to-noise ratio of up to 4–6—although on about one-third of the total number of transits the signal was weaker than 4σ —but for two days (12 April 1997 and 23 May 1997) the signal was very strong ($>10\sigma$) and wide ($>80 \text{ ms}$). This procedure has been tested and found to work satisfactorily during our generic weak pulsar survey (V.M.M., O.I.M. and N. Shegoleva, unpublished data), which concentrated especially on short-period pulsars.

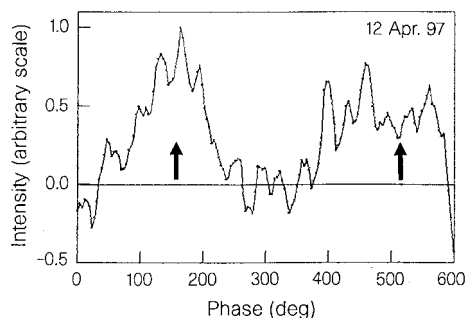


Figure 2 Pulse profile obtained when the observing window equals twice the apparent pulsar period ($P = 474$ ms), this profile was obtained by the integration of 477 independent individual pulses. The two pulse profiles are one period apart. Expected positions of pulses are shown by two arrows. The data in this window were then folded with the apparent pulsar period to get the mean profile for one period. This profile is given as the bottom panel in Fig. 1.

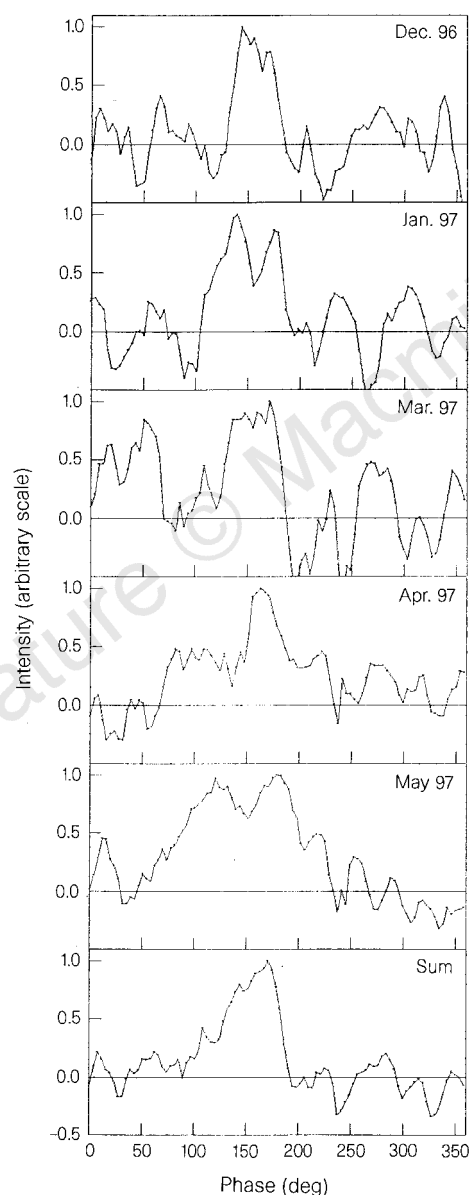


Figure 3 Mean pulse profiles obtained as the sum of various observing periods. Top to bottom, sum of: 4 days containing 3,180 individual pulses (December 96); 4 days and 3,816 pulses (January 97); 10 days and 9,540 pulses (March 97); 7 days and 6,678 pulses (April 97); 5 days and 4,293 pulses (May 97). The bottom panel gives the mean pulse profile of all the preceding five panels (27,507 pulses).

for reduction. The profile is much wider in the fifth example in Fig. 1. We saw this type of broad profile during five out of 30 days. Figure 1 shows that the integration of 954 individual pulses is not enough to obtain a stable profile shape, but this figure also shows that, on all five days, the profiles appear at the same phase, giving independent support to the reality of the signals.

Further evidence of the presence of pulsed emission is given in Fig. 2, where we show the pulse profile obtained by integrating the signals at twice the pulsar period (474 ms). The profile is clearly double.

If we add the measurements in each of the observing epochs and average all available measurements (30 days), we find the mean profile shown in Fig. 3; the signal-to-noise ratio of the mean profile is now more than nine. Again it can be seen that the integration of a few thousand pulses is not enough to obtain the stable profile shape. The pulse width at half maximum, $W_{0.5}$, varies between 10 and 80 ms. The mean value of $W_{0.5}$ is 42 ± 21 ms, corresponding to 64° or 177 milliperiods. The ratio $W_{0.5}/P$ seems to be large compared to most 'normal' radio pulsars at this frequency²¹. It is also large compared to the same ratio obtained from the radio profiles (J. H. Taylor *et al.*, unpublished data) from the six known γ -ray pulsars²². The pulse shape has a width greater than the γ -ray emission profile⁶ and is more like the X-ray emission⁵ profile. We could not detect the interpulse seen in the γ -ray emission⁶.

On the basis of the TOA of 11 strong records, a timing solution has been obtained during a span of 153 days of observations: epoch (JD) = 2450434.434; $P = 0.23709938265 (\pm 29)$ s; $\dot{P} = 10.88 (\pm 5) \times 10^{-15} \text{ s s}^{-1}$. Our data are consistent with the results of other workers (J. H. Taylor *et al.*, unpublished data) at the 2σ level.

We estimated the flux density of Geminga for each of the 37 days that observations were obtained by use of known calibration sources. The mean value for the flux S , averaged over the pulsar period, is 60 mJy with $\sigma = 95$ mJy. There are, however, large variations apparent from day to day ($S = 5\text{--}500$ mJy), as expected for such a nearby object owing to scintillation in the interstellar medium. If the radiation is highly polarized, we would in addition expect to see large variations, as the LPA uses only one linear polarization. We cannot exclude strong intrinsic variations of the radio emission.

We may compute the spectral index of the radio emission, if we take the upper limit at 1.4 GHz (ref. 15), $S \leq 1$ mJy, as second reference point. The spectral index α is computed from the dependence $S(\nu) \propto \nu^{-\alpha}$, where ν is the frequency. The resulting spectral index, $\alpha \geq 1.6$, is in good agreement with the values found usually for pulsars²³. If Geminga has a steeper spectral index, for example, like the Crab pulsar ($\alpha = 3.1$), it is clear why this object has not been detected above 102 MHz.

It is also possible to estimate the luminosity at 400 MHz (L_{400}). We use the mean spectral index $\alpha = 1.9$ for class S pulsars with simple and steep spectra²³; this class of pulsars includes other 'multi-frequency pulsars' such as the Crab and Vela pulsars. We obtain $L_{400} = 0.1 \text{ mJy kpc}^2$, which is the smallest luminosity value for all known radio pulsars (J. H. Taylor *et al.*, unpublished data). $\square$

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A single-electron transistor made from a cadmium selenide nanocrystal

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The techniques of colloidal chemistry permit the routine creation of semiconductor nanocrystals^{1,2} whose dimensions are much smaller than those that can be realized using lithographic techniques^{3–6}. The sizes of such nanocrystals can be varied systematically to study quantum size effects or to make novel electronic or optical materials with tailored properties^{7–9}. Preliminary studies of both the electrical^{10–13} and optical properties^{14–16} of individual nanocrystals have been performed recently. These studies show clearly that a single excess charge on a nanocrystal can markedly influence its properties. Here we present measurements of electrical transport in a single-electron transistor made from a colloidal nanocrystal of cadmium selenide. This device structure enables the number of charge carriers on the nanocrystal to be tuned directly, and so permits the measurement of the energy required for adding successive charge carriers. Such measurements are invaluable in understanding the energy-level spectra of small electronic systems, as has been shown by similar studies of lithographically patterned quantum dots^{3–6} and small metallic grains¹⁷.

Fig. 1a shows an idealized diagram of the device. CdSe nanocrystals 5.5 nm in diameter^{18,19} are bound to two closely spaced gold leads by bifunctional linker molecules^{20,21}. The leads are fabricated on a degenerately doped silicon wafer, which is then used as a gate to tune the charge state of the nanocrystal under study. Figure 1b shows a field-emission scanning electron micrograph of a completed device. A number of nanocrystals seem to be in the ~5 nm gap between the electrodes.

Most devices, including the particular junction shown in Fig. 1b, have immeasurably high impedance ($R > 100 \text{ G}\Omega$). Only about 1 in 20 have a measurable conductance, typically in the range 10 M Ω to 1 G Ω . Devices of this type typically behave as though transport is occurring through a single nanocrystal¹¹, as we demonstrate below. This may initially seem surprising, as Fig. 1b indicates that the number of nanocrystals in the junction region is quite large. However, tunnelling through the linker molecules has an exponential decay length of less than 1 Å (refs 11, 20, 22). As a result, only a well-placed nanocrystal (within 2 nm of each lead) can contribute to conduction.

Figure 2 shows the linear response conductance, G , of a device measured at $T = 4.2 \text{ K}$ as a function of the gate voltage, V_g , applied to the substrate. As the gate bias is raised, the conductance grows to a peak and then declines to zero again. In the insets to Fig. 2 the current, I , flowing through the nanocrystal is plotted as a function of the voltage, V , applied between the two leads at two fixed values of V_g . The I – V taken at a V_g away from the linear response peak shows

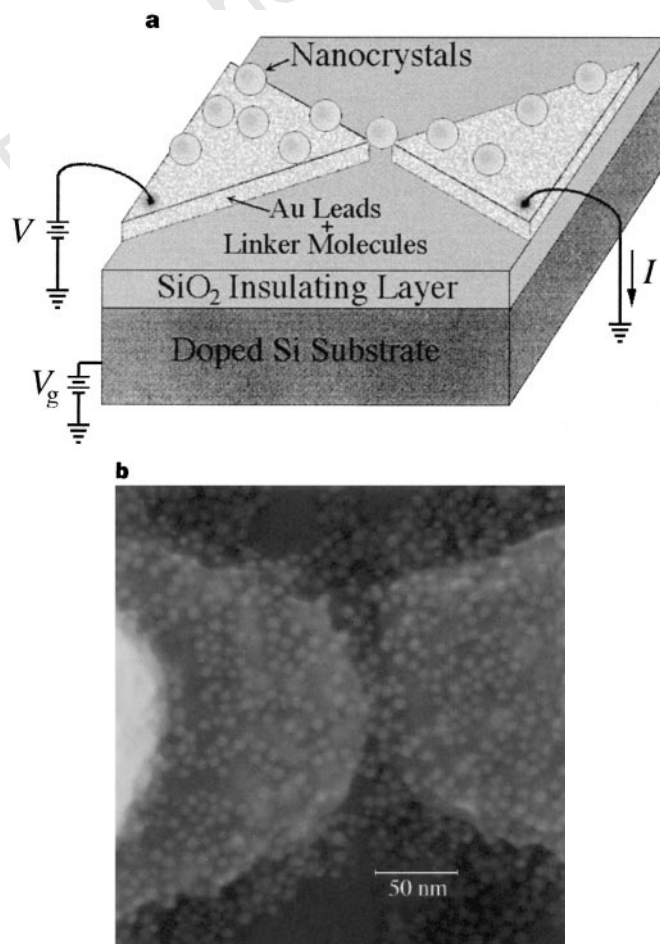


Figure 1 a, Diagram of the device. Nanocrystals sit on top of two closely spaced leads. Transport measurements probe the nanocrystal that best bridges the gap between the leads. The electrochemical potential of this nanocrystal can be tuned by applying a gate voltage to the substrate. **b**, Field-emission scanning electron micrograph of a set of 13-nm-thick leads separated by a ~10 nm gap onto which CdSe nanocrystals 5.5 nm in diameter have been deposited. A combination of optical and electron-beam lithography coupled with shadow evaporation is used to define these leads. The leads are patterned on a degenerately doped wafer on which an oxide barrier 53 nm thick has been grown. The nanocrystals have a mean diameter of 5.5 nm and a 5% coefficient of variation and are attached to the leads with 1,6-hexanedithiol. One end of these linear dithiol molecules binds to gold leads and the other binds to the nanocrystals to form a 1.2-nm-thick insulating barrier. Further fabrication details are described in ref. 11.

a suppressed conductance at small V whereas the I - V characteristic taken at the centre of the peak has a finite linear conductance for small V . Figure 3 shows a map of the differential conductance of the device, plotted as a grey scale, as a function of both V and V_g .

These measurements can be understood by using ideas borrowed from studies of lithographically patterned dots^{3,5,6}. The conductance peak in Fig. 2 is a Coulomb oscillation. The suppressed conductance on each side of the peak is a consequence of the finite energy required to add (remove) an electron to (from) the nanocrystal in its ground state. This energy is analogous to the electron affinity (ionization energy) of a molecule. The peak occurs when the two charge states of the nanocrystal have the same total (including electrostatic) energy and an extra electron can therefore hop on and off the nanocrystal at no energy cost. The maximum size of the gap in the I - V curves away from the peak provides a measure of the addition (removal) energy for electrons, as we discuss below.

Figure 4a is a schematic energy level diagram of the nanocrystal with N electrons at a gate voltage midway between two Coulomb oscillations. The electrochemical potential for adding the $(N+1)$ th electron to the nanocrystal is denoted μ_{N+1} . The energy difference Δ_{N+1} between μ_{N+1} and μ_N is referred to as the addition energy. In the simplest model, referred to as the Coulomb blockade model⁶, the addition energy is given by:

$$\Delta_{N+1} = \mu_{N+1} - \mu_N = U + \Delta E$$

where U is the Coulomb interaction energy between any two electrons on the nanocrystal and $\Delta E = E_{N+1} - E_N$ is the energy-level spacing to the next unoccupied single-particle eigenstate.

When the linear-response conductance is zero, a finite V can adjust the Fermi energies of the leads relative to the nanocrystal to add or remove an electron to or from the nanocrystal. The Fermi level to the left lead, equal to $\sim eV$, can either rise above μ_{N+1} or fall below μ_N , allowing current to flow. The range of V for which the conduction is zero is referred to as the Coulomb gap. This gap varies as a function of V_g . The maximum size of the Coulomb gap for N electrons on the nanocrystal, in other words the maximum voltage V that can be applied without current flow, is a direct measurement of the addition energy Δ_{N+1} for the next electron. This is illustrated in Fig. 4b.

The evolution of the Coulomb gap with V_g can be seen in Fig. 3a and b, where the differential conductance dI/dV is plotted as a

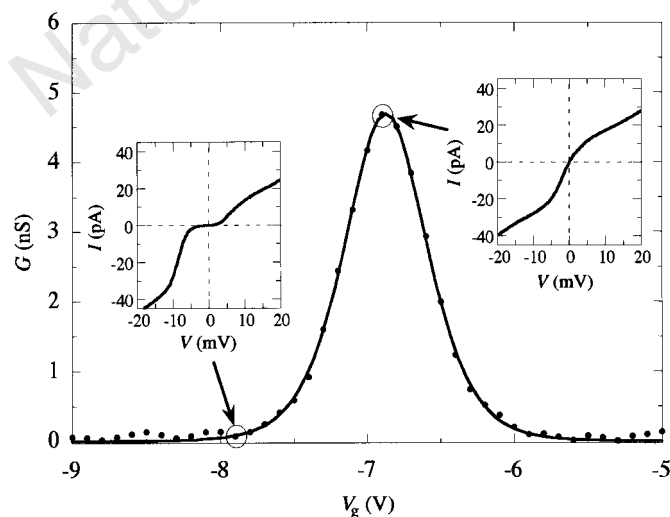


Figure 2 Conductance, G , plotted against gate voltage, V_g , for a single-nanocrystal transistor measured at $T = 4.2$ K. The conductance shows a peak when the charge state of the nanocrystal changes by one electron. The dots are the measured values; the solid curve is a fit to the data by using the standard Coulomb blockade model⁶ with a temperature $T = 5$ K. Insets: I - V characteristics measured at the gate voltages indicated.

function of both V_g and V . The Coulomb gap produces the light-coloured diamond-shaped regions in these figures. The Coulomb gap is zero at a Coulomb oscillation; it grows to a maximum size approximately halfway between two oscillations, and then decreases again to zero at the next Coulomb oscillation. Figure 3c shows a schematic evolution of the Coulomb gap, along with the inferred addition energies, Δ_N , for successive electrons. These energies range from ~ 15 to 30 meV.

Similar, although less complete, results have also been obtained in two other gated samples. In addition, I - V measurements on eight other samples without a gate have been performed, some of which have been presented previously¹¹. The addition energies determined for these samples are typically between 30 and 60 meV.

In the Coulomb blockade model, the addition energy for adding successive carriers is $U + \Delta E$. A straightforward electrostatic estimate for U , including the screening effects of the metallic leads, yields $U \sim 50$ meV for a 5.5 -nm-diameter metallic sphere¹¹. The level spacing, ΔE , depends on the type of charge carriers and is predicted to be ~ 100 meV for electrons and ~ 10 meV for holes²³. The measured values of 15 – 60 meV for the addition energies are thus consistent with, but somewhat smaller than, expectations (~ 50 – 60 meV) for individual holes on a CdSe nanocrystal.

We now address an intriguing aspect of the data. Note that on the

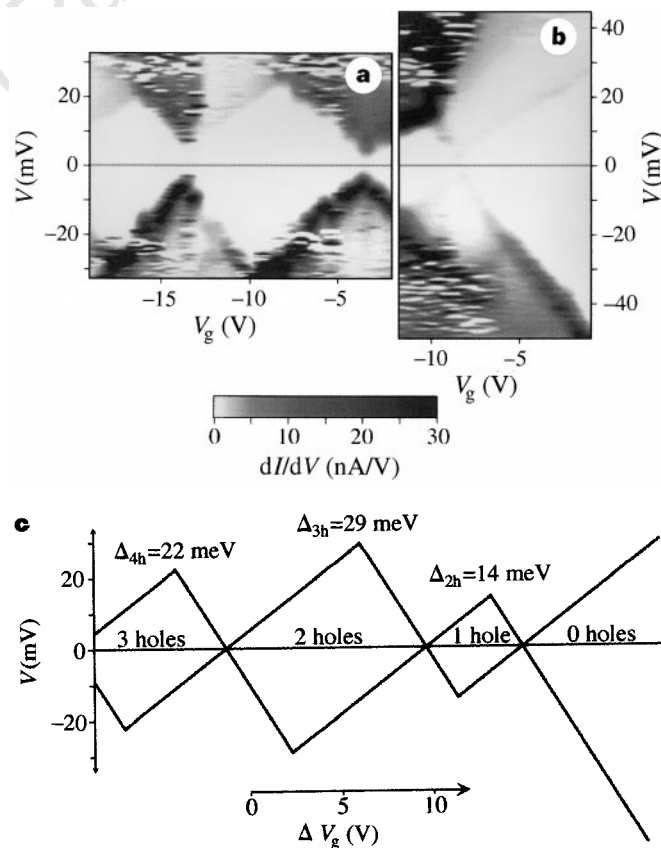


Figure 3 a, b, Composite grey-scale plots of the differential conductance dI/dV of a CdSe nanocrystal plotted as a function of both V_g and V . The white diamond-shaped regions correspond to the Coulomb gap. During the course of acquiring these data, several 'switches' occurred where the entire characteristic shifted along the V_g axis. These switches are ubiquitous in Coulomb blockade measurements and are thought to result from sudden changes in the local electrostatic environment. One such change occurred between the acquisition of **a** and **b**. During the acquisition of **a**, two smaller shifts occurred. To account for this, some parts of the data have been translated along the V_g axis. **c**, A schematic illustration of the data from **a** and **b**, indicating the inferred number of holes on the nanocrystal as a function of V_g and the addition energy for successive holes.

right side of Fig. 3 the Coulomb gap continues to grow with increasingly positive V_g . For even larger gate voltages than shown in the figure (up to 40 V) the Coulomb gap exceeds 150 mV and there is no evidence for any more Coulomb oscillations.

This behaviour can be understood if electrons are being added to the valence band of the nanocrystal with increasingly positive V_g . In this case, for some sufficiently positive V_g , every state in the valence band is filled, and the next extended state of the nanocrystal lies in the conduction band, 2 eV higher in energy. In this interpretation, the large gap at positive V_g corresponds to a completely filled valence band. Starting on the right side of Fig. 3 and decreasing V_g , the first Coulomb oscillation of Fig. 3 then corresponds to the removal of the first electron from, or alternatively the addition of the first hole to, the valence band. A further decrease in V_g adds additional holes. The addition energies for the second, third and fourth holes are $\Delta_2 = 14 \pm 2$ meV, $\Delta_3 = 29 \pm 3$ meV and $\Delta_4 = 22 \pm 2$ meV, as is indicated in Fig. 3c.

Note that in the Coulomb blockade model, the energy Δ_2 , for adding the second hole to the nanocrystal is simply the Coulomb interaction, U , between holes, as the lowest-lying hole state is doubly degenerate. The energy, Δ_3 , for adding the third hole, in contrast, is $U + \Delta E$, where ΔE is the difference between the ground and first excited single-particle state. The addition energy for the fourth hole, Δ_4 , is again U because the first excited state is also doubly degenerate. The results given above are somewhat consistent with this scheme: for example, Δ_3 , predicted to be $U + \Delta E$, is greater than Δ_2 , which is predicted to be U .

Nevertheless, discrepancies remain. The energy for adding the second hole, $\Delta_2 = 14$ meV, is significantly smaller than the values of $U \sim 50$ mV obtained from simple electrostatic estimates or from measurements of other samples. In addition, the measured Δ_4 is greater than Δ_2 , whereas in the Coulomb blockade model they should be the same. The origin of these differences remains unclear, although the effects of exchange and correlations are expected to be important for these few-hole systems. We also emphasize that the large gap at positive V_g has been seen in only one device. To confirm that this gap is associated with a filled valence band and to verify the inferred hole addition energies, it will be necessary to repeat these measurements on other samples.

This work represents a new type of spectroscopy for single nanocrystals. Unlike optical measurements, where electron-hole pairs are created, these measurements probe the energy for adding a single type of charge carrier. We hope that these results will stimulate new calculations of the hole addition energies for a

CdSe nanocrystal that include the effects of exchange, correlations, and screening by the metallic electrodes. Future measurements will investigate how the ground-state and excited-state properties vary with the size, shape and composition of nanocrystals as well as with the composition of the leads. □

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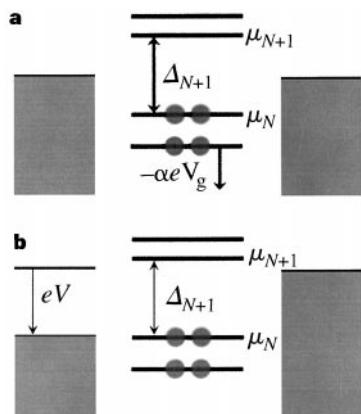


Figure 4 **a**, Energy-level diagram for a nanocrystal with N electrons at a gate voltage midway between two Coulomb oscillations. The electrochemical potential of the N -electron nanocrystal is denoted μ_N , and the electrochemical potential of the $(N + 1)$ -electron nanocrystal is denoted μ_{N+1} . **b**, The application of a finite bias, V , can overcome the Coulomb gap. The maximal V applied before conduction occurs is equal to the addition energy, Δ_{N+1} for the $(N + 1)$ th electron.

A tough SiAlON ceramic based on α - Si_3N_4 with a whisker-like microstructure

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Silicon nitride (Si_3N_4) is a light, hard and strong engineering ceramic^{1,2}. It can withstand harsh environments and support heavy loads at temperatures beyond those at which metals and polymers fail. It can also be manufactured reliably at a reasonable cost and in large quantities. There are two forms of silicon nitride³: α - Si_3N_4 and β - Si_3N_4 . The former is harder, but only

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the latter is currently used in engineering applications, because only this form can be given a microstructure resembling a whisker-reinforced composite^{1,2,4}, which gives it the necessary toughness and strength. Here we report the synthesis of a tough α - Si_3N_4 solid solution with this kind of microstructure. This material is 40% harder than β - Si_3N_4 and is equally strong and tough. Its hardness (22 GPa) is exceeded only by boron carbide and diamond (which are both brittle). These properties mean that this form of α - Si_3N_4 should be preferred over β - Si_3N_4 for all engineering applications, and it should open up new potential areas in which the ceramic can be applied.

Si_3N_4 is inherently strong because of the covalent chemical bond between Si and N. α - Si_3N_4 has a structure similar to that of β - Si_3N_4 , containing SiN_4 tetrahedra forming a corner-shared three-dimensional network with the characteristic (001) plane of a hexagonal

structure³. This is also the plane of fast growth in β - Si_3N_4 . The stacking of the planes is different in α and β structure; it is ABCD in α - Si_3N_4 and AB or CD in β - Si_3N_4 . The longer stacking sequence in α - Si_3N_4 is responsible for its higher hardness because of the longer Burgers vector required for slip dislocations⁵.

α - Si_3N_4 is unstable relative to β - Si_3N_4 and converts to the latter at higher temperature. This turns out to be useful. If α - Si_3N_4 is used as a starting powder and slowly converted to β - Si_3N_4 in the presence of a sintering, bonding liquid of silicon oxynitride, the newly grown crystals that consume the unstable matrix tend to take the shape of long rods in much the same way as crystals found in geological formations. The resultant β - Si_3N_4 has a microstructure that mimics a log-jam or pressed wood, but on a much finer scale. It contains hexagonal rods of single crystals as thin as several micrometres, bonded together and reinforcing each other. This makes β - Si_3N_4

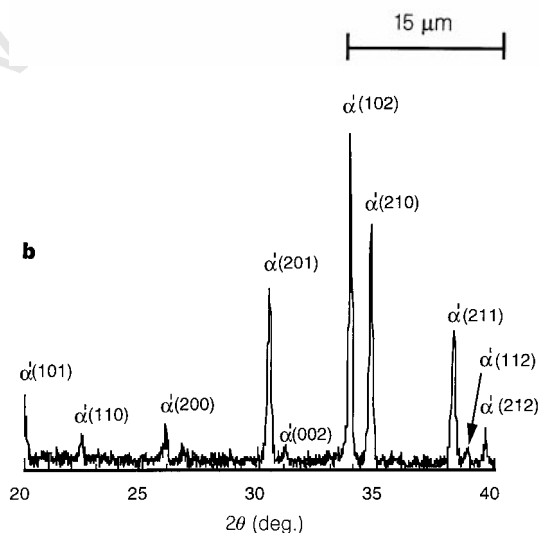
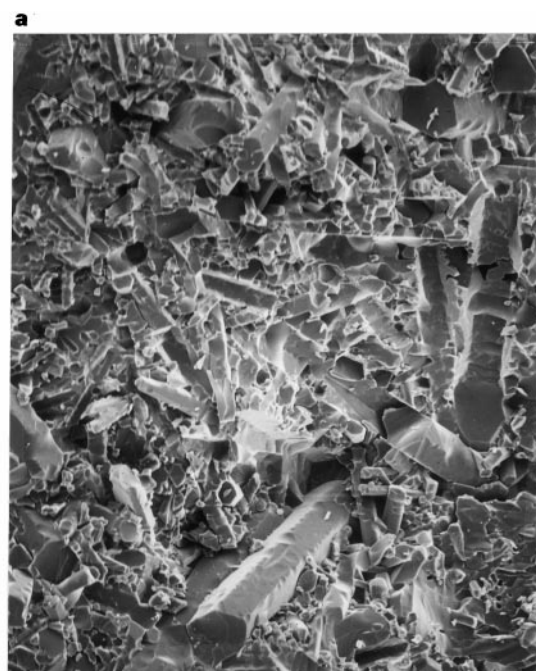


Figure 1 Properties of an $\text{Nd}_{0.4}\text{Si}_{9.9}\text{Al}_{2.1}\text{O}_{0.9}\text{N}_{15.1}$ sample. **a**, Scanning electron micrograph of a fracture surface showing elongated grains. **b**, X-ray diffraction pattern of **a** showing single-phase α' -SiAlON reflections ($a = 7.8140 \text{ \AA}$, $c = 5.6914 \text{ \AA}$). Fired at $1,950^\circ\text{C}$ for 1.5 h.

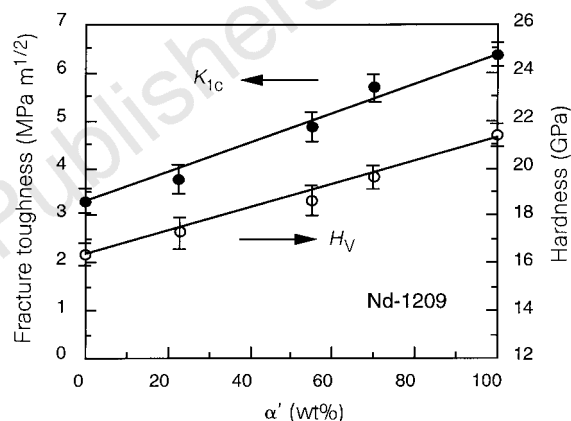


Figure 2 Fracture toughness (K_{1c}) and hardness (H_V) of $\text{Nd}_{0.4}\text{Si}_{9.9}\text{Al}_{2.1}\text{O}_{0.9}\text{N}_{15.1}$ (known as Nd-1209) samples with different amounts of α' -SiAlON; the remainder is untransformed β - Si_3N_4 . Both properties increase with the amount of $\beta \rightarrow \alpha'$ transformation. Using the nomenclature described in the text, $m = 1.2$ and $n = 0.9$ in Nd-1209.

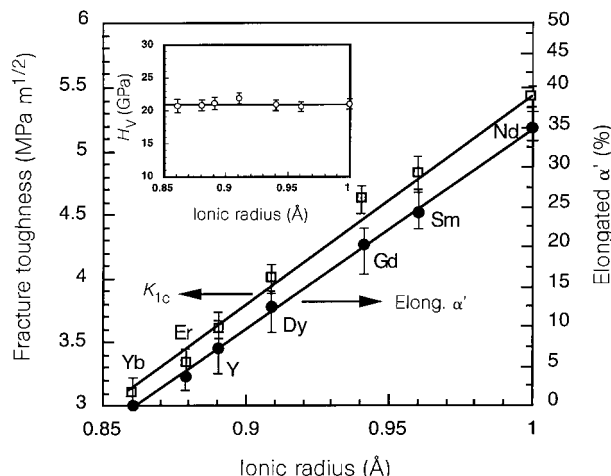


Figure 3 Fracture toughness (K_{1c}) and amount of elongated grains of seven α' -SiAlONs stabilized by cations of different ionic radii for an overall composition of $\text{R}_{0.4}\text{Si}_{9.6}\text{Al}_{2.4}\text{O}_{12}\text{N}_{14.8}$ ($m = 1.2$, $n = 1.2$; see text). The amount of elongated α' grains was measured by stereology (that is, by counting their projected areas on the photograph of the fracture surface). The inset shows a nearly constant hardness (H_V) value for all samples.

difficult to break, hence its higher strength and toughness. We have known for 30 years that if β - Si_3N_4 is used as a starting powder, it is difficult to obtain the above microstructure and the high strength and toughness^{4,6,7}. Indeed, without the whisker-like microstructure, high toughness and high strength would be very difficult to achieve in any covalent ceramic⁸, even though it may possess extraordinary hardness and oxidation resistance^{9,10}.

Can the same microstructure be obtained for α - Si_3N_4 , or its cation-stabilized solid solution known as α' -SiAlON? This solid-solution has a general formula $\text{M}_{m/2}\text{Si}_{12-m-n}\text{Al}_{m+n}\text{O}_n\text{N}_{16-n}$ where M is a cation of charge z that enters the large interstitial site available in α - Si_3N_4 (ref. 11). Crystallographically, the hexagonal (001) plane and the prismatic (210) plane have essentially the same atomic structures in both α - and β - Si_3N_4 . Therefore, the growth habit and the microstructure should be similar. Nevertheless, despite numerous attempts to produce α' -SiAlON with a whisker-like microstructure, few have succeeded and none have reported improved fracture toughness^{12–18}. For example, after a systematic study of α' -SiAlONs, Nordberg *et al.*¹⁸ recently concluded that “no systematic variation of mechanical properties with the overall size and shape of the α' -SiAlON grains could be discerned”. They also found liquid-rich compositions promoting the formation of elongated α' -SiAlON grains¹⁷. This, however, is undesirable because of the poor mechanical and chemical stability of the liquid-rich SiAlONs at high temperature^{1,2}.

It is notable that in all the previous studies only α - Si_3N_4 was used as starting material. On the other hand, the fact that self-reinforced β - Si_3N_4 composed of elongated crystals can be readily obtained by using α - Si_3N_4 as a starting powder suggests that the reverse scheme (using β - Si_3N_4 as a starting powder) could be successful if self-reinforced α' -SiAlON is to be obtained. This is indeed the case, as illustrated by the following examples.

We prepared samples of Nd-stabilized α' -SiAlON using β - Si_3N_4 powder (SNP21FC, Denka Inc.; the powder contains 7% α - Si_3N_4). The microstructure contains many elongated grains with sharp, crystallographic facets (Fig. 1a). The X-ray diffraction pattern of this specimen (Fig. 1b) confirms that only reflections of α' -SiAlON exist. This material is both hard and tough, with an indentation

hardness of 21.7 GPa and an indentation toughness of $6.3 \text{ MPa m}^{1/2}$. Using identical measuring methods, a self-reinforced β - Si_3N_4 showed a hardness of 15.5 GPa and a toughness of $6.2 \text{ MPa m}^{1/2}$. Thus, the self-reinforced α' -SiAlON is 40% harder than β - Si_3N_4 and is equally tough. In comparison, a variety of equiaxed α' -SiAlONs made from α - Si_3N_4 powder showed a typical hardness ranging from 20.5 to 22.0 GPa and a toughness of $3.0 \text{ MPa m}^{1/2}$. The three-point bend strengths of the self-reinforced α' -SiAlON samples exceeded 700 MPa, comparable to those of self-reinforced β - Si_3N_4 prepared in our laboratory but twice the values of α' -SiAlON made from α - Si_3N_4 powder. Also, compared with SiC (another engineering ceramic), the new α' -SiAlON is as hard but has improved strength and toughness.

The conversion from β - Si_3N_4 to α' -SiAlON is relatively slow in the above material. We followed the conversion in different samples and found it to have a direct effect on the properties. As shown in Fig. 2, there is a monotonic increase in both hardness and toughness with the amount of α' -SiAlON. The increase in hardness with the amount of α' -SiAlON is well known^{19,20}. However, all previous reports^{19,20} have indicated that the toughness decreases with the amount of α' -SiAlON. As the only difference between our materials and those studied in the past lies in the microstructure, the reason our materials behave differently is that the α' -SiAlON grains grown here are all elongated.

Elongated grains in β - Si_3N_4 are thought to form because of slow nucleation⁷. When α - Si_3N_4 powder is used to make β - Si_3N_4 , only a small number of β - Si_3N_4 grains initially exist or are subsequently nucleated. These relatively few grains can then grow considerably in the (001) direction before they run into each other. Presumably, the same applies to the $\beta \rightarrow \alpha'$ transformation when β - Si_3N_4 powder is used. However, the formation of elongated α' grains is not always guaranteed by using β - Si_3N_4 powder. When the composition favours the formation of α' -SiAlON so much that the conversion of β - Si_3N_4 is too fast, the α' -SiAlON obtained still has fine equiaxed grains. This may be attributed to the overabundance of nucleation. An illustration of this trend is seen by comparing $\text{R}_{0.4}\text{Si}_{9.6}\text{Al}_{2.4}\text{O}_{1.2}\text{N}_{14.8}$ compositions using different rare earths (R). At 1,950 °C, all of these compositions yield 100% α' -SiAlON. Yet the microstructures of these samples are different, ranging from equiaxed grains (R is Yb) to elongated grains (R is Nd). Figure 3 shows their hardness and toughness, along with the percentage of elongated α' -grains. Obviously, only the amount of elongated α' -SiAlON is responsible for the toughness enhancement.

The systematic difference in the microstructure of different rare-earth-stabilized α' -SiAlONs is related to their phase stability. According to the phase diagram²¹ shown in Fig. 4, the α' -region of Yb- α' is much larger than that of Nd- α' at the same temperature. Thus, Yb- α' -SiAlON is more stable and the driving force of $\beta \rightarrow \alpha'$ transformation is higher. This results in a higher nucleation rate and a finer grain size. This different phase stability is no doubt related to the ionic size of the stabilizing cations, as recently verified by molecular-orbital calculations²².

If overabundance of nucleation is the problem, it should be possible to circumvent it by reacting at a lower temperature that provides slower kinetics and lower driving force²¹. In the $\text{Yb}_{0.4}\text{Si}_{9.6}\text{Al}_{2.4}\text{O}_{1.2}\text{N}_{14.8}$ composition, for example, we effected slower nucleation of α' -SiAlON by first holding it at 1,550 °C for 1.5 h (material B). This contrasts with the fine equiaxed grains obtained after direct firing at 1,950 °C (material A). Likewise, if the same composition is held at 1,650 °C for 12 h, the conversion to α' -SiAlON is also slowed and the final microstructure consists of elongated grains (material C). The properties of these three materials of identical compositions but different processing steps are summarized in Table 1 and show that, by controlling α' nucleation, very substantial enhancement of toughness can be achieved for Yb- α' -SiAlON. These Yb- α' -SiAlONs are extremely stable. For example, after holding at 1,500 °C for 240 h, the X-ray diffraction patterns

Table 1 Properties of $\text{Yb}_{0.4}\text{Si}_{9.6}\text{Al}_{2.4}\text{O}_{1.2}\text{N}_{14.8}$ fired in three different ways

Firing	Elongated α' (%)	H_V (GPa)	K_{1c} ($\text{MPa m}^{1/2}$)
A	0	20.8 ± 0.3	3.1 ± 0.3
B	20	21.1 ± 0.8	4.8 ± 0.4
C	25	21.4 ± 0.5	5.1 ± 0.4

H_V , hardness; K_{1c} , fracture toughness; see text for description of firings A, B and C.

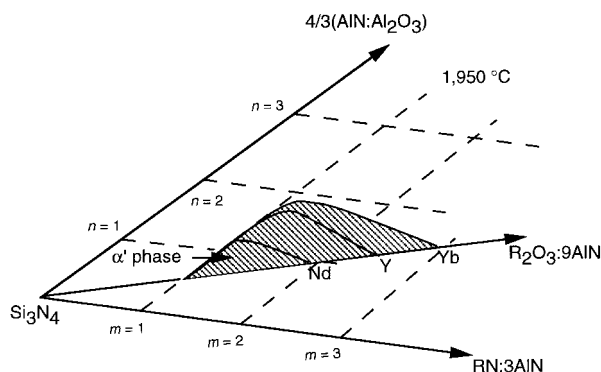


Figure 4 α' phase region in the Si_3N_4 corner of the phase diagram of (Si, Al, R) (N, O) system at 1,950 °C (ref. 21). It is smaller for larger stabilizing cations and lower temperatures. m and n are defined in the text; R is the rare-earth stabilizing element.

of these materials show only α' -SiAlON reflections in a pattern identical to that obtained before the treatment of 1,500 °C. Using other similar ways of controlling nucleation, we have been able to obtain high-toughness single-phase α' -SiAlON with rare-earth or Y stabilizer over a broad range of compositions²¹. □

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Synthesis of microporous transition-metal-oxide molecular sieves by a supramolecular templating mechanism

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Mesoporous bulk^{1–4} and thin-film^{5–7} silicates with pore sizes of 20–100 Å can be synthesized by using micellar aggregates of long-chain organic surfactant molecules as templates to direct the structure of the silicate network. Because of the potential applications of these molecular-sieve materials as catalysts, separation membranes and components of sensors, it is desirable to extend the range of accessible pore sizes and material compositions. Mesoporous oxides in which transition metals partially⁸ and fully^{9–13} substitute for silicon have been made by similar means, in the latter case by ensuring strong interactions between the

surfactants and the transition-metal alkoxide precursors. Templating with organic molecules has also been long used for the synthesis of microporous materials—synthetic zeolites—which have smaller pore sizes (4–15 Å), but here the organic molecules are shorter-chain amphiphiles which are too small to be considered true surfactants and so act as discrete entities around which the framework crystallizes^{14–16}. Here we show that even such short-chain molecules can aggregate into supramolecular templates when they form bonds with transition-metal (niobium) alkoxides, and that in this way they can direct the formation of transition-metal oxides with pore sizes of less than 20 Å. These pore sizes, which result from the smaller diameter of micellar structures of the short-chain amines relative to the longer-chain surfactants used for the synthesis of mesoporous materials, qualify the resulting molecular sieves as microporous, even though the supramolecular templating mechanism is similar to that used to make the mesoporous materials. Thus our approach extends the supramolecular templating method to afford microporous transition-metal oxides.

In a typical synthesis, 3 g of Nb(OC₂H₅)₅ was hydrolysed in 41 g of deionized water to give a suspension of loosely bound niobium oxy-alkoxide precipitates. 0.96 g of hexylamine was then introduced as a templating agent to form an organic–inorganic nanocomposite. The nanocomposite was hydrothermally treated to promote condensation and crystallization. The aged product was recovered by filtration and dried at room temperature. Figure 1 shows the X-ray diffraction (XRD) patterns of niobium oxide–hexylamine nanocomposites produced by different ageing temperatures. For comparison, a product prepared by direct hydrolysis of niobium ethoxide without the addition of hexylamine was examined. Figure 1, trace a, shows that the material prepared without a templating agent was amorphous, having no distinct XRD peaks. With hexylamine addition, a broad, low-intensity (100) diffraction peak was found after ageing the material at ambient conditions for 24 hours (Fig. 1 trace b). After the ageing temperature was raised to

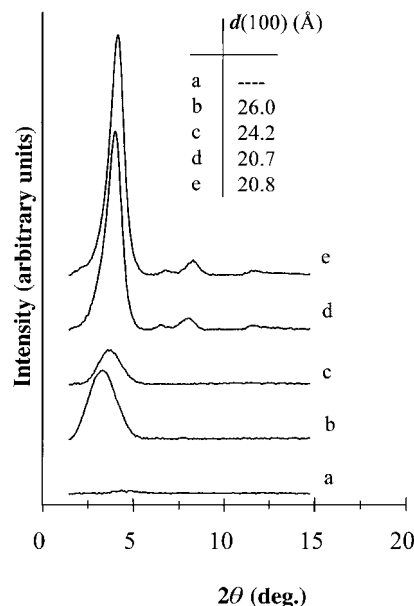


Figure 1 X-ray diffraction (XRD) patterns of as-prepared niobium oxide samples derived from hydrolysis of Nb(OC₂H₅)₅. Trace a, sample prepared without amine templating agent; traces b–e, samples prepared with subsequent hexylamine addition (Nb:amine molar ratio of 1:0.75). The samples were examined after ageing at 25 °C for 24 h (a, b), 96 °C for 24 h (c), 180 °C for 24 h (d), and 180 °C for 48 h (e). The XRD patterns were obtained with a Siemens D5000 θ – θ diffractometer with CuK α radiation (λ = 1.5418 Å).

96 °C, the (100) peak was shifted to higher 2θ angle (Fig. 1 trace c). When the ageing temperature was increased to 180 °C, three additional peaks were noted, corresponding to the (110), (200) and (300) diffractions of a hexagonal phase (Fig. 1 trace d). Prolonged ageing at 180 °C produced even more crystalline products (Fig. 1 trace e).

The XRD patterns show that our niobium oxide–hexylamine nanocomposite has a hexagonally packed structure similar to the mesoporous MCM-41^{1–4,8,17,18} and TMS1^{9–13}, except that the $d(100)$ -spacing of our materials is much smaller owing to the smaller supramolecular template size. We have also successfully removed these short-chain amine templating agents, by washing the nanocomposite powder with a water–alcohol solution containing nitric acid, to produce microporous niobium oxide molecular sieves, termed Nb-TMS5, that retain a hexagonal crystal structure to ~400 °C. The transmission electron microscopy (TEM) image (Fig. 2) of Nb-TMS5 appears to feature well-aligned planes with detailed texture in the form of discrete bright spots, illustrating the presence of well packed micropores. The microporosity of Nb-TMS5 is further illustrated by the type I N_2 adsorption and desorption isotherms shown in Fig. 3. Hexane and mesitylene were also used in sorption experiments to characterize the pore

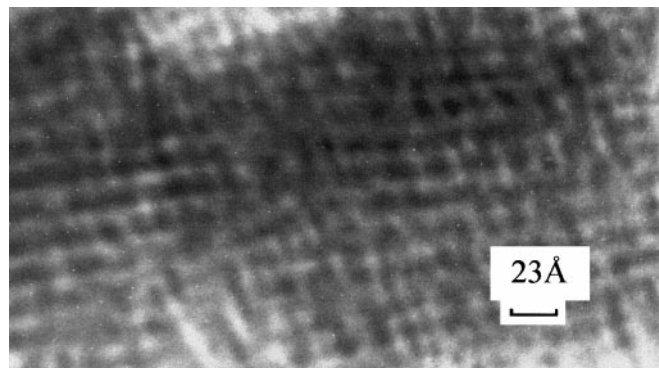


Figure 2 TEM micrograph of Nb-TMS5 synthesized with a Nb:hexylamine molar ratio of 1:0.75, and aged at 180 °C. The image was obtained with a 200 kV JEOL 002B instrument.

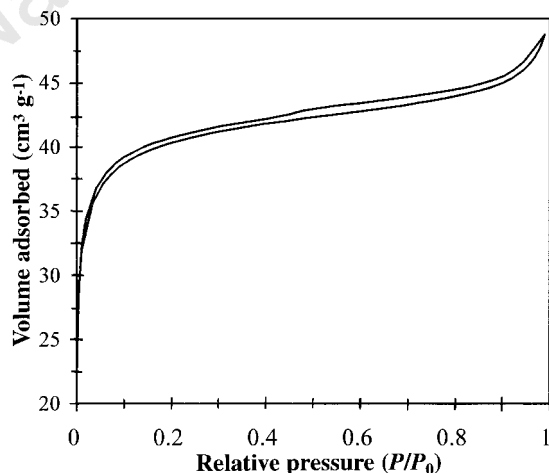


Figure 3 N_2 adsorption and desorption isotherms of microporous Nb-TMS5 synthesized with a Nb:hexylamine molar ratio of 1:0.75 and aged at 180 °C. The isotherms were collected at STP on a Micromeritics 2010 Gas Adsorption Analyzer after the sample was washed with acidic ethanolic solution and degassed at 180 °C at 1 μ torr for 12 h.

size of this Nb-TMS5 sample, and both gave the same pore volume, indicating the pore size is >8.5 Å (the critical molecular diameter of mesitylene). Although the upper limit of the pore size could not be precisely determined by sorption methods owing to difficulties in handling larger hydrocarbon molecules, the N_2 adsorption/desorption isotherm clearly showed the absence of mesopores, which was confirmed by TEM. We also found that, depending on the detailed conditions for ageing and amine removal, the Brunauer–Emmett–Teller surface area obtained for Nb-TMS5 synthesized with hexylamine may vary from 120 to 250 m² g^{−1}.

We have found that our supramolecular templating approach for making well-defined microporous Nb-TMS5 can be broadly applied to other short-chain amine molecules. Figure 4 shows that the $d(100)$ -spacing of these materials can be systematically varied between 17.7 and 25.1 Å by increasing the hydrocarbon chain

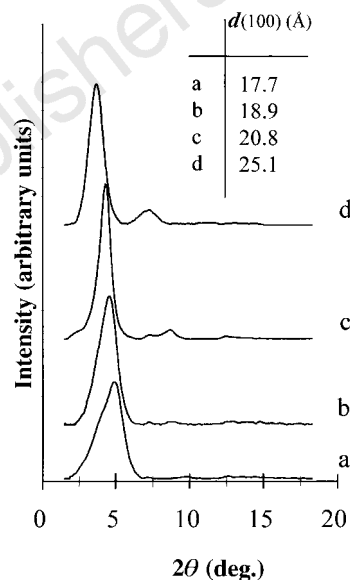


Figure 4 Representative powder X-ray diffraction patterns of Nb-TMS5 synthesized with butylamine (trace a), amylamine (b), hexylamine (c) and heptylamine (d) as a templating agent.

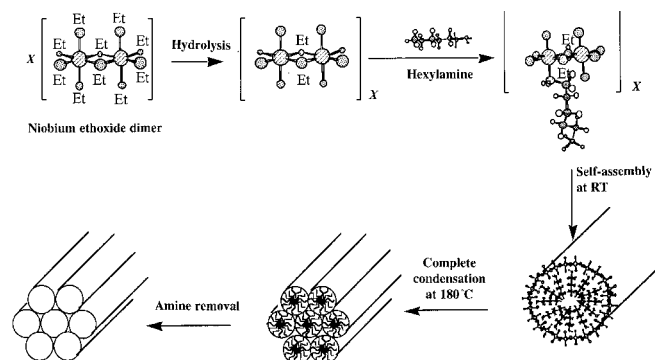


Figure 5 Schematic illustration of the Nb-TMS5 synthesis: (1) partial hydrolysis of niobium ethoxide at low temperatures; (2) introduction of hexylamine; (3) self-assembly of hexylamines as supramolecular templates; (4) condensation and crystallization of inorganic framework at high temperatures; and (5) amine removal by acidic washes.

length of the amines from 4 to 7. This ability to tailor a well-defined pore size in the microporous regime should be useful in shape- and size-selective molecular sieving applications as it bridges the gap in the application of templating approaches to the synthesis of structures with pore dimensions of <20 Å.

Microporous zeolites (ZSM-5) are obtained in place of mesoporous MCM-41 when silica gels with tetradecyltrimethyl ammonium bromide surfactants are hydrothermally aged at temperatures above 150°C (ref. 3). This suggests that at high temperatures even large surfactant molecules will not be able to direct mesoporous structure formation, because of unfavourable conditions for micelle formation. Our present results, however, clearly demonstrate that even at 180°C , synthesis with much smaller organic templating agents generates hexagonally packed microporous transition metal oxides (TMS5), instead of a transition-metal-oxide analogue of ZSM-5 zeolites. Although the small molecules we have employed (butylamine, amylamine, hexylamine and heptylamine) are often used as discrete molecular templates in directing zeolite synthesis, we find that, with the proper interaction with the metal oxide precursor, they can aggregate into supramolecular templates like the larger surfactant molecules employed in MCM-41 and TMS1 synthesis.

Unlike the mesoporous MCM-41 and TMS1 syntheses, our approach has used a partially hydrolysed niobium alkoxide gel that had been prepared before the addition of organic templating agents. Such a preparation precludes the formation of micellar phases in the precursor medium. In another experiment, we observed that when water was added to an ethanolic mixture of niobium ethoxide and hexylamine, a material having a XRD pattern similar to that of TMS5 could be produced. This second approach is similar to the procedure adopted by Antonelli and Ying^{9,10} for synthesizing hexagonally packed mesoporous Nb-TMS1. In this case, a discrete covalent bond was used to direct the templating interaction between the organic and inorganic phases. To ensure the formation of the niobium-amine surfactant complex, niobium ethoxide was mixed with amine surfactant before the addition of water. Our success in producing Nb-TMS5 by these two different synthetic procedures suggests that pre-hydrolysis of the alkoxide precursor does not prevent the proper establishment of a strong interaction between the niobium oxide precursor and the amine molecules, as required for self-assembly. We propose that the low-temperature hydrolysis of niobium alkoxide created only a loosely bound oxy-alkoxide gel that could restructure by redissolution and peptization on introduction of the templating agents.

It is critical to ensure proper interaction of the organic and inorganic phases in the nanocomposite at low temperatures. This may proceed as illustrated in Fig. 5. The hydrophobic nature of the hydrocarbon tail forces the small amine molecules to self-assemble, forming a hydrophobic core. Ageing above room temperature facilitates hydrolysis of the bulky ethoxy groups, and shrinkage of the micellar core occurs. Further assembly to form well-defined hexagonally packed structures of niobium oxide-hexylamine nanocomposites requires hydrothermal ageing at temperatures above 100°C . The high-temperature treatment promotes the formation of well crystallized structures as well as the complete polycondensation of the inorganic network. Following such a preparation, the amine templating agents can be removed without damaging the inorganic framework to yield stable microporous niobium oxides. We note that a strong interaction between the niobium and amine molecules occurred in both of our synthesis procedures. As in the case of mesoporous Nb-TMS1, washing with ethanol at 100°C was not a viable approach for removing the templating agents in Nb-TMS5; the strong bonding between the amine and Nb was broken only by protonating the head group of the organic molecule through acidic washes. This bonding between the niobium precursor and the amine template distinguishes our approach from the MCM-41 synthesis with ammonium bromide¹⁵ and hydrogen-bonded neutral

surfactants⁸, in which the templating molecules could be easily removed from the silica framework by alcoholic washes. We have also found that only an amorphous niobium oxide gel was obtained if we used short-chain molecules which do not form strong bonding with Nb (such as hexane, hexanol or hexanone) in our syntheses instead of hexylamine; in such cases the pH of the suspension was adjusted by the addition of other bases to give the same value as was found when using hexylamine. □

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A synthetic peptide ligase

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The preparation of synthetic molecules showing the remarkable efficiencies characteristic of natural biopolymer catalysts remains a formidable challenge for chemical biology. Although significant advances have been made in the understanding of protein structure and function, the *de novo* construction of such systems remains elusive^{1–5}. Re-engineered natural enzymes and catalytic antibodies, possessing tailored binding pockets with appropriately positioned functional groups, have been successful in catalysing a number of chemical transformations, sometimes with impressive efficiencies^{6–11}. But efforts to produce wholly synthetic catalytic peptides have typically resulted in compounds with questionable structural stability, let alone reactivity¹. Here we describe a 33-residue synthetic peptide, based on the coiled-coil structural motif^{12–14}, which efficiently catalyses the condensation of two shorter peptide fragments with high sequence- and diastereoselectivity. Depending on the substrates used, we observe

rate enhancements of tenfold to 4,100-fold over the background, with catalytic efficiencies in excess of 10^4 . These results augur well for the rational design of functional peptides.

Coiled-coils are assemblies of two or more α -helical peptides which wrap around each other with a slight left-handed superhelical twist. The sequences of these peptides generally show a seven-residue repeat ($abcdefg$)_n, in which the first and fourth positions are typically occupied by hydrophobic amino acids and the fifth and seventh by charged amino acids^{15–19} (Fig. 1). The identity of these amino acids has been shown to determine the stability and the aggregation state of the coiled-coil ensemble. The catalyst **E** used in this study is a 33-residue polypeptide with valine and leucine residues at positions *a* and *d*, respectively, and lysines at *e* and *g* positions of the heptad repeat. We hypothesized that the hydrophobic surface presented by the catalyst **E** in the α -helical conformation would provide sufficient thermodynamic driving force for the binding and structural preorganization of two shorter complementary fragments **S**₁ and **S**₆. Furthermore, complementarity between the charged residues at position *e* and *g* of the catalyst and the substrates was expected not only to provide the specificity in substrate recognition and catalysis, but also disfavour homo-assemblies of the catalysts and substrates owing to charge–charge repulsion^{15–19}. We anticipated that built-in electrostatic interactions would be an essential design feature for preventing catalyst self-aggregation and thus the inhibition that has plagued related studies^{4,5}. Given appropriate activation of the substrates (Fig. 2), the rate of the condensation reaction was expected to be significantly enhanced owing to a higher effective concentration of the

substrates within the ternary complex **E**·**S**₁·**S**₆ (Fig. 3).

Typically, condensation reactions were initiated by adding a solution of the electrophilic fragment **S**₁ to a buffered solution (pH 7.5) of the nucleophile (see Methods). Owing to the efficiency of the catalyst, product formation was too rapid ($>1 \mu\text{M s}^{-1}$) to follow conveniently at ambient temperatures. Therefore, the initial rates were measured at 5 °C. We observed rate enhancements up to 4,100-fold compared with the uncatalysed background coupling in the reaction of equimolar mixtures of **S**₁ and **S**₆ ($[\text{S}_1] = [\text{S}_6] = 200 \mu\text{M}$) containing various amounts of the catalyst **E** (11–430 μM) (Fig. 4a). Experiments in which the electrophilic substrate **S**₁ or the nucleophilic substrate **S**₆ were replaced with the variants **S**₃ (hydroxamic acid and **S**₉ (glycine in place of the amino-terminal cysteine residue) showed no catalytic activity, reinforcing the expected importance of carboxy-terminal activation and the cysteine moiety for the coupling process. In contrast, no detectable difference in the rate of product formation was observed if **E**^{*}, in which the cysteine residue is replaced by a serine, was used in place of **E**, indicating that the cysteine residue of the catalyst does not play a special role in the ligation process.

The **P**^{*} → **P** rearrangement for the catalysed reaction is relatively slow ($t_{1/2} > 1 \text{ min}$), resulting in an initial build-up of **P**^{*} in the reaction mixture. This contrasts sharply with the uncatalysed reaction, in which **P**^{*} can not be detected^{4–5,20–23}. The isolated thioester **P**^{*} (in the absence of **E**) is stable under acidic conditions but rearranges instantly upon addition of MOPS buffer (pH 7.5). In MOPS buffer containing hydroxylamine (0.5 M) the hydrolysed electrophilic peptide fragment, the corresponding hydroxamic acid,

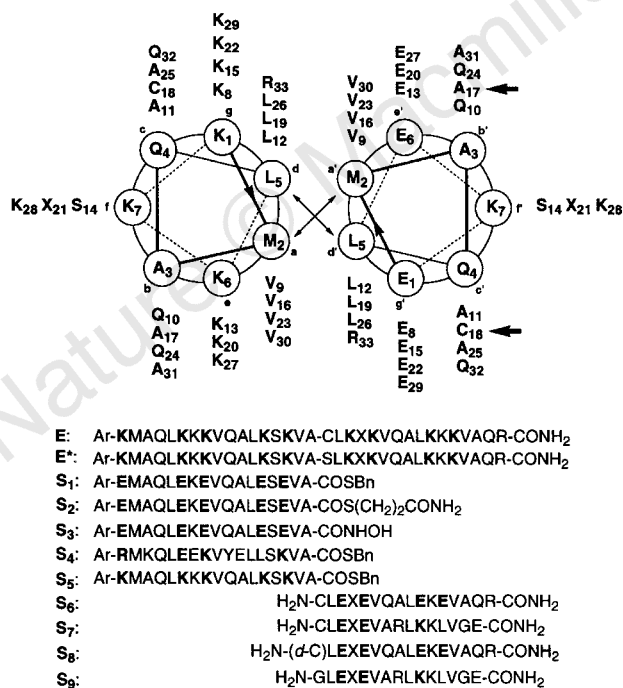


Figure 1 Helical-wheel diagram of the catalyst **E** bound to its substrates **S**₁ and **S**₆, along with the sequences of the peptides used in these experiments. The complementary electrostatic interface, formed by the lysine and glutamic acid residues at the *e* and *g* positions (shown in bold), assures specific catalyst–substrate binding and helps to minimize catalyst self-inhibition. To allow sensitive monitoring of product formation by high-performance liquid chromatography (HPLC), the spectroscopic label 4-acetamido-benzoic acid was coupled to the N termini of **S**_{1–5} and **E** (denoted Ar) and to Lys 22 side-chains of **S**_{6–9} and **E** (denoted X). The methods and techniques used to synthesize and characterize the peptides used in this study have been described elsewhere⁵. Arrows indicate the alanine and cysteine residues critical for the ligation process.

Table 1 Sequence selectivity for the catalytic process

Reaction	Observed rate enhancements
S ₁ + S ₆	1,800 (4,100)*
S ₁ + S ₈	180
S ₁ + S ₇	82
S ₄ + S ₆	9.2
S ₅ + S ₆	1

Reactions performed as described in Methods, using 50 mol% catalyst.
 * Rate enhancement in the presence of 215 mol% catalyst.

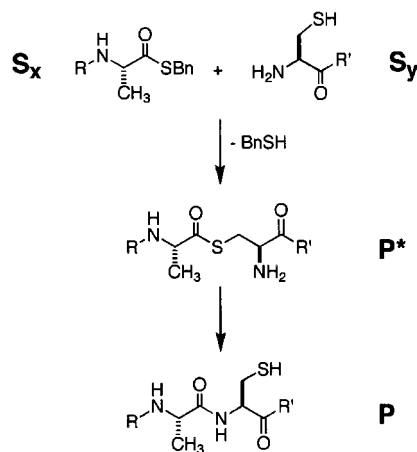


Figure 2 The amide-bond-forming process based on Kent strategy²⁰. The fragment ligation proceeds through an initial trans-thioesterification reaction between the activated C terminus of the electrophilic substrate **S**_x and the N-terminal cysteine side-chain functionality of the nucleophilic substrate **S**_y to produce the corresponding intermediate thioester **P**^{*}, which rapidly rearranges to give the final native peptide **P** (refs 20, 30, 31).

and the nucleophilic peptide fragment S_6 are obtained, confirming the thioester structure of the intermediate P^* (ref. 24). These results suggest that the rearrangement is inhibited by the catalyst E , most likely because of reduced conformational freedom of P^* in the $E \cdot P^*$ complex (Fig. 3). Nonlinear least-squares fitting of the experimental data to this model (assuming single turnover) using the program SimFit provided the following approximate values: $k_{cat} = k_3 = 1 \times 10^{-2} s^{-1}$, $k_{uncat} = k_1 = 1 \times 10^{-3} M^{-1} s^{-1}$ and $K_{E \cdot S_1 \cdot S_6} = 10^{-7} M^2$ yielding $(k_{cat}/K_{E \cdot S_1 \cdot S_6})/k_{uncat} = 10^4$ (see Fig. 3 for definitions). The observed catalytic efficiency of the 33-residue peptide compares favourably to that of the two catalytic antibodies^{25–27} selected for amino-acid couplings but lags behind that of engineered subtilisin ligases²⁸.

The formation of structurally organized substrate–catalyst aggregates such as $E \cdot S_1 \cdot S_6$ is supported by the results of circular dichroism (CD) measurements using unreactive analogues (Fig. 5). As expected, both the catalyst and the substrates are predominantly

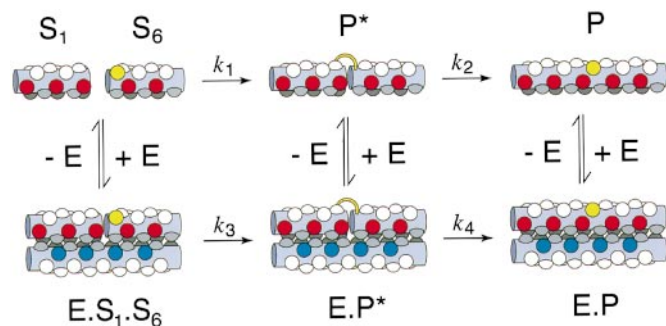


Figure 3 Minimal reaction model for the catalytic process. The negatively charged electrophilic and nucleophilic peptide substrates S_1 and S_6 are preorganized on the complementary, positively charged catalyst E , forming the ternary complex $E \cdot S_1 \cdot S_6$ (with Michaelis constant $K_{E \cdot S_1 \cdot S_6}$). This ensemble facilitates the condensation reaction (k_3) owing to a higher effective molarity of the reactants. Rate-limiting rearrangement (k_4) of the resulting thioester complex $E \cdot P^*$ gives heterodimeric coiled-coil $E \cdot P$, dissociation of which regenerates the catalyst E . The template-independent background reaction is shown above (k_1, k_2). The rearrangement of $E \cdot P^*$ is slow in comparison to that of the single-stranded thioester P^* ($k_4 < k_2$). Peptide backbones are shown as cylinders and side chains as spheres. Residues critical for molecular recognition and ligation are shown coloured (red, glutamic acid; blue, lysine and arginine; yellow, cysteine; grey, leucine and valine).

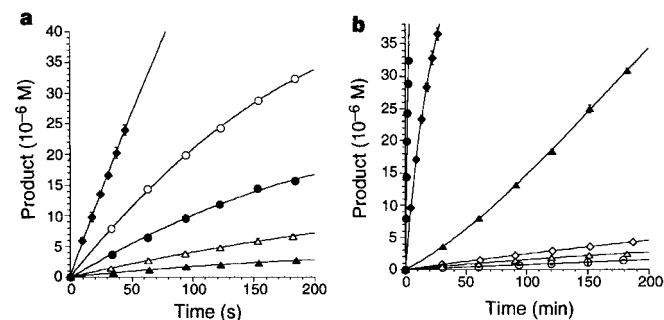


Figure 4 Experiments showing catalysis of peptide coupling reactions. **a**, Product formation (P) as a function of time for reaction mixtures initially containing 200 μM S_1 , 200 μM S_6 and 11 μM ($\blacktriangle$), 24 μM ($\triangle$), 53 μM ($\bullet$), 104 μM ($\circ$), and 430 μM ($\blacklozenge$) catalyst E , respectively. **b**, Production formation ($P^* + P$) as a function of time for reactions containing equimolar mixtures of the peptide fragments S_1 and S_6 ($\circ$), S_4 and S_6 ($\triangle$), and S_1 and S_7 ($\diamond$) ($[S_1] = [S_7] = 200 \mu M$). In the presence of 50 mol% catalyst rate enhancements of 9.2-fold (S_4 and S_6 , $\blacktriangle$), 82-fold (S_1 and S_7 , $\blacklozenge$), and 1,800-fold (S_1 and S_6 , $\bullet$) over the uncatalysed reaction are observed.

random-coil at concentrations of 50 μM . However, CD spectra of equimolar mixtures of the components indicate a small but significant amount of α -helical induction in accordance with the formation of heteromeric complexes.

If catalysis of bimolecular reactions is based on tight binding of substrates in close proximity, product inhibition is often a problem owing to the inherent increase in binding affinity of the catalyst for the product²⁹. This inhibition is also reflected in the growth profiles of the present ligase system: a pronounced curvature is observed on saturation of the catalyst with the product. However, the viability of catalytic turnover was demonstrated in reactions between S_2 and S_6 ($[S_2] = [S_6] = 200 \mu M$, pH 5.0). The less reactive alkylthioester of S_2 allowed these experiments to be performed at room temperature. In the presence of 18 μM catalyst, 27 μM of product was produced after 20 h, whereas only 1 μM was formed over the same time period in the uncatalysed reaction.

The high sequence- and diastereoselectivity of the 33-residue ligase was revealed by experiments with altered electrophilic (S_4, S_5) and nucleophilic (S_7) peptide fragments (Fig. 1). These new peptide sequences were designed with the same amino-acid composition in the hydrophobic interface, but different polar residues in positions e and g of the heptad repeat. When condensation reactions between S_4 and S_6 or S_1 and S_7 ($[S_x] = 200 \mu M$) in the presence of 50 mol% catalyst were analysed a pronounced specificity emerged: the ligation between S_4 and S_6 shows a 9.2-fold rate enhancement, whereas the ligation between S_1 and S_7 displays a 82-fold rate enhancement (Fig. 4b). These results contrast with the 1,800-fold rate enhancement observed for reactions between S_1 and S_6 under similar catalyst concentrations (Table 1). The precipitous decrease in rate with increasing number of electrostatic mismatches (one for S_7 , two for S_4) suggests a cooperative destabilizing effect of electrostatic mismatches. Indeed, catalysis is absent in reactions with the substrate S_5 , which possesses the same hydrophobic sequence as S_1 but which has exclusively non-complementary residues in the e and g positions. The importance of electrostatic interactions to the catalytic process is further highlighted by the fact that the catalytic efficiency is strongly dependent on salt concentration: when the reaction between S_1 and S_6 is performed in the presence of 500 mM NaCl ($[S_1] = [S_6] = 200 \mu M$; $[E] = 50 \mu M$) the initial rate of product formation is reduced by a factor of 4 in comparison to the reactions without NaCl. Thus the overall efficiency of the catalytic process is highly dependent on the electrostatic contributions from the residues in the e and g position, in spite of the fact that these positions lie on the exposed edges of the helices which might have been predicted to have weak electrostatic contributions owing to

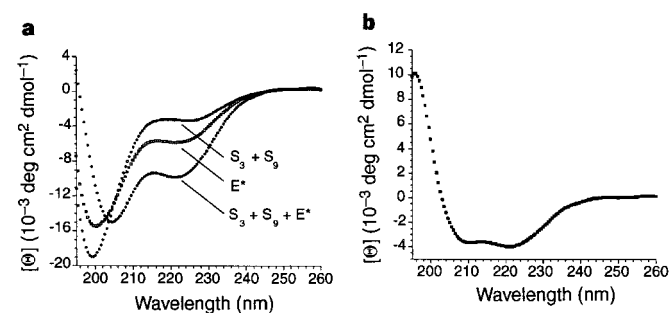


Figure 5 Circular dichroism (CD) spectra supporting the formation of organized substrate–catalyst aggregates. **a**, CD spectra of the catalyst E^* , a mixture of substrates S_3 and S_9 , and a mixture of S_3, S_9 and E^* . All fragments were 50 μM in 10 mM pH 7.5 phosphate buffer. To prevent fragment condensation reactions and adventitious disulphide formation, E^* and S_9 , in which the cysteine residues were replaced with serine and glycine, respectively, and S_3 , in which the S_1 thioester was converted to the unreactive hydroxamic acid equivalent, were used. **b**, The difference CD spectrum obtained by subtraction of the spectra of the substrates and catalyst from the spectrum of the mixture.

favourable interactions with the polar aqueous solution. The sensitivity of the coupling reaction rate to small changes in the aggregate stability may provide a tool for elucidating the fine-structure/stability relationships of heterodimeric coiled-coils.

Precise structural organization at the active site is believed to be of pivotal importance for successful catalysis in enzymes as well as catalytic antibodies. The ordered nature of the ligation site in the artificial ligase described here was further demonstrated by control reactions with the diastereoisomeric peptide fragment S_8 , in which the N-terminal L-cysteine was replaced with D-cysteine. Direct competition experiments between S_6 and S_8 with equimolar amounts of S_1 ($[S_1] = [S_6] = [S_8] = 200 \mu\text{M}$; $E = 100 \mu\text{M}$) revealed a pronounced selectivity (10:1) for the 'natural' ligation product **P**, which presumably reflects the strict conformational requirements for the fragments at the reaction site.

This study demonstrates the use of α -helical protein-like assemblies in artificial enzyme design. It remains to be seen whether more complex active sites can also be rationally engineered. □

Methods

The reactions were performed in 0.6-ml Eppendorf tubes. Temperature was maintained at $5.0(\pm 0.5)^\circ\text{C}$. Benzylmercaptan (1 μl) was added to a degassed solution containing MOPS buffer (pH 7.50), the nucleophilic peptide fragment S_x , the catalyst **E**, and the internal standard 4-acetamidobenzoic acid (ABA); the mixture was incubated for 30 min. Reactions were initiated by adding a solution containing the electrophilic peptide fragment S_y to give a total volume of 330 μl . Typically, the following concentrations were obtained: $[S_x] = [S_y] = 200 \mu\text{M}$, $[\text{MOPS}] = 100 \text{ mM}$, $[\text{ABA}] = 100 \mu\text{M}$. All experiments were repeated twice. Samples (50 μl) were removed from the reaction vessel at various time points, immediately quenched with 5% trifluoroacetic acid (40 μl), and stored at -78°C before HPLC analysis.

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Severe chemical ozone loss in the Arctic during the winter of 1995–96

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Severe stratospheric ozone depletion is the result of perturbations of chlorine chemistry owing to the presence of polar stratospheric clouds (PSCs) during periods of limited exchange of air between the polar vortex and midlatitudes and partial exposure of the vortex to sunlight^{1–4}. These conditions are consistently encountered over Antarctica during the austral spring. In the Arctic, extensive PSC formation occurs only during the coldest winters, when temperatures fall as low as those regularly found in the Antarctic^{1,5,6}. Moreover, ozone levels in late winter and early spring are significantly higher than in the corresponding austral season^{1,7,8}, and usually strongly perturbed by atmospheric dynamics^{9–12}. For these reasons, chemical ozone loss in the Arctic is difficult to quantify. Here we use the correlation between CH₄ and O₃ in the Arctic polar vortex to discriminate between changes in ozone concentration due to chemical and dynamical effects¹⁰. Our results indicate that 120–160 Dobson units (DU) of ozone were chemically destroyed between January and March 1996—a loss greater than observed in Antarctica in 1985, when the 'ozone hole' was first reported^{13,14}. This loss outweighs the expected increase in total ozone over the same period through dynamical effects, leading to an observed⁶ net decrease of about 50 DU. This ozone loss arises through the simultaneous occurrence of extremely low Arctic stratospheric temperatures^{6,15} and large stratospheric chlorine loadings. Comparable depletion is likely to recur because stratospheric cooling^{16,17} and elevated chlorine concentrations^{5,18} are expected to persist for several decades.

In this study, we use observations of CH₄, O₃ and HCl made by the Halogen Occultation Experiment (HALOE)¹⁹ on board the Upper Atmosphere Research Satellite (UARS). HALOE does not

cover the Arctic vortex regularly. However, on many occasions in the winter of 1995–96, solar occultation measurements were obtained in vortex air, characterized by high potential vorticity and low methane mixing ratio, the latter indicating diabatic descent¹⁰. To quantify chemical ozone loss we consider the relationship between O₃ and the practically chemically inert tracer CH₄ (ref. 10). Inside the vortex, this relation can not be altered by transport processes because of the limited exchange of air between vortex and mid-latitudes²⁰. Therefore, this relationship should, in the absence of chemical processes, be preserved throughout the lifetime of the vortex, that is, during winter and spring^{10,20}. This is indeed observed for HALOE measurements of the relationship between the two chemically inert compounds HF and CH₄ throughout the lower stratosphere. Thus, any deviation from the initial CH₄/O₃ (and likewise CH₄/HCl) relationship over the winter must be due to chemical change.

The variations of the volume mixing ratios of both O₃ and HCl with respect to that of CH₄ inside the Arctic vortex over the winter 1995–96 are shown in Fig. 1. For a given observed CH₄ mixing ratio, throughout the lower stratosphere, much lower HCl values are measured in January and early March than in November (Fig. 1 bottom panel), indicative of substantial conversion of HCl to chemically more active forms. This observation is in accordance with measurements of strongly enhanced ClO concentrations in the vortex from 21 December 1995 to 3 March 1996 by the Microwave Limb Sounder (MLS)²¹.

Extremely low HCl mixing ratios are observed inside the vortex in early March 1996, consistent with the occurrence of a PSC event on 3 March 1996²¹. HCl increases thereafter and reaches a maximum concentration in mid-April. HCl mixing ratios seen in April are, however, still below those observed in November 1995, before PSC processing. This observation is consistent with the timescale for HCl

recovery after PSC processing in the Arctic vortex being greater than one month^{4,22,23}.

The O₃/CH₄ relationship remains unchanged between November 1995 and January 1996 (Fig. 1 top panel, black and orange symbols), that is, over the time period when not enough sunlight is available to initiate chemical ozone depletion. Towards the end of the lifetime of the vortex between early March and mid-April 1996, however, a substantial fraction of ozone has been chemically destroyed (Fig. 1 top panel, red and purple symbols). Comparing ozone mixing ratios of 'early' and 'late' vortex measurements for equal values of the CH₄ mixing ratio, ozone reductions are evident in the height range 12–22 km (corresponding to potential temperature $\Theta = 350$ –590 K and a CH₄ mixing ratio of 0.4–1.5 p.p.m.v.). This is consistent with substantial ozone losses inferred from lidar measurements²⁴, MLS observations⁶ and sonde data²⁵ collected during the same period (that is, late winter 1995/96). The HALOE measurements of March and early April 1996 (Fig. 1 top panel), indicate that chemical destruction results in local ozone losses of 60% over the height range 14–18 km ($\Theta = 400$ –480 K and a CH₄ concentration of 0.7–1.1 p.p.m.v.); the most severe depletion of ozone of up to 3.3 p.p.m.v. (>70%) is observed at ~17 km (450 K). These large losses persist throughout the observation period until 10 April 1996.

The simultaneous measurements of CH₄ and O₃ can be further exploited to estimate the ozone mixing ratios expected in the vortex in the absence of chemical ozone removal^{10,20}. As a reference state for the conditions before the onset of chemical perturbations (see also Fig. 1) we take the O₃/CH₄ relationship observed in late November 1995 in the incipient vortex, identified by potential vorticity and diabatic descent¹⁰:

$$\text{O}_3 = 2.98 (\text{CH}_4)^3 - 11.20 (\text{CH}_4)^2 + 9.52 (\text{CH}_4) + 2.14 \quad (1)$$

where O₃ and CH₄ concentrations are in p.p.m.v. (this is valid for

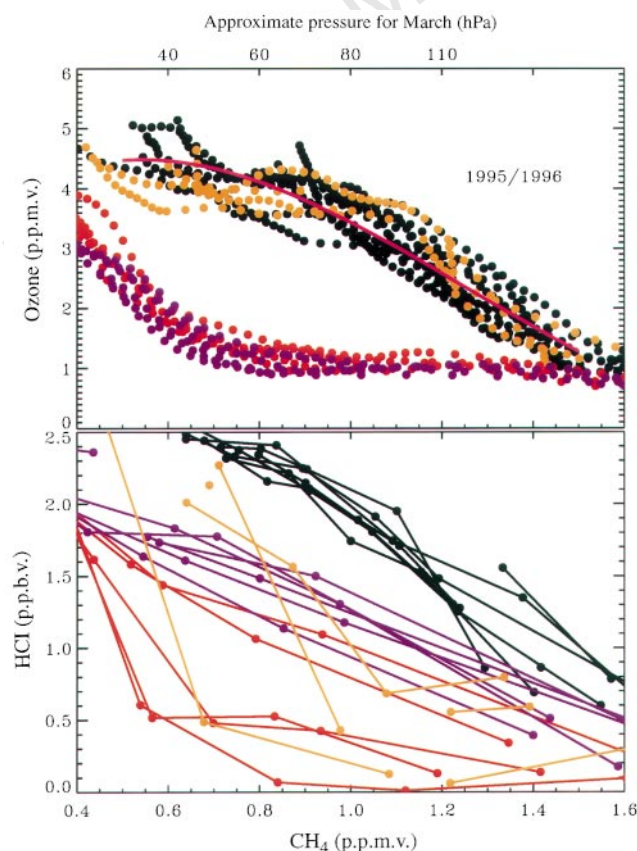


Figure 1 The relationship between CH₄ volume mixing ratios and those of O₃ (top panel) and HCl (bottom panel) in the stratosphere inside the Arctic polar vortex for the winter of 1995–96. Approximate pressure levels for the March observations are given on the top axis. Black symbols indicate measurements in November (measured at 51–46° N, for an average potential vorticity at 550 K of 59.9 p.v.u.), orange symbols in late January (50–48° N, potential vorticity at 550 K 60.5 p.v.u.), red symbols measurements in March (54–70° N, potential vorticity at 550 K of 99.7 p.v.u.), and purple symbols measurements in early April (70–59° N, potential vorticity at 550 K of 89.2 p.v.u.). (1 p.v.u. = 10⁻⁶ K m² (kg s)⁻¹.) An empirical 'early vortex' relation between O₃ and CH₄ (equation (1)) is shown as a solid purple line (the variation in the November observations introduces an uncertainty in the proxy ozone of ~0.3 p.p.m.v.). All HALOE observations inside the vortex are shown for November and January; for March and April only a representative selection of observations is shown for better readability. Data outside the pressure range 10–150 hPa and data points outside the vortex are excluded. All data are processed with HALOE software version 18.

0.5 p.p.m.v. < CH₄ < 1.6 p.p.m.v.). Inside the vortex, throughout its existence, this relation (equation (1)) should remain unaltered in the absence of chemical processes²⁰. Therefore, the measurements of CH₄ in March and April 1996 may be combined with the reference relationship (equation (1)) to derive a proxy for the ozone mixing ratio that would be expected for March and April in the absence of chemical change¹⁰. The separation between these proxy ozone profiles $\hat{O}_3$ (Fig. 2 green symbols) and the ozone profiles measured in March and early April in the vortex (Fig. 2 red symbols) is a measure of the accumulated ozone reduction between November and early spring. A severe ozone reduction is discernible throughout the lower stratosphere. Moreover, the Arctic ozone profiles in early 1996 are very close to those measured in austral spring 1985 at the Georg Forster station²⁶.

The change in column ozone in the lower stratosphere between late November and March/early April attributed to chemistry (Fig. 2) has been calculated¹⁰ from individual HALOE profiles in the vortex during March and April 1996 between 12 and 21 km (Θ = 350–550 K; pressure, 150–25 hPa; CH₄ mixing ratio, 0.5–1.5 p.p.m.v.). Because most ozone resides in this altitude region, the calculated chemical loss provides a good approximation for the loss in the total ozone column caused by chemical reactions involving anthropogenic chlorine¹⁰. All measurements inside the vortex in late winter and spring 1996 indicate chemical removal of column ozone exceeding 100 DU, with typical values for March and April 1996 ranging between 120 and 160 DU (Table 1).

As a result of chemical loss and dynamical resupply, the ozone column (above 100 hPa) observed by HALOE decreases from about 250 DU in late November 1995 to about 200 DU in March/April 1996 (Table 1). This reduction by ~50 DU is similar to that measured by MLS⁶. Combined with the calculated estimate of the chemical ozone loss, this implies that without chemical change, the total ozone column would have increased by the order of 100 DU over this period. Such an increase is indeed consistent with the climatology of total ozone in high northern latitudes as observed before the appearance of anthropogenic chlorine in the stratosphere^{7,8,27}. Furthermore, the derived ozone column losses are consistent with the deviation of the Arctic total ozone values in March/April 1996 from their climatological mean of 435 ± 50 DU (ref. 27).

For all six winters of HALOE observations in the Arctic, substantial halogen-catalysed ozone depletion in late March and early April was observed (Table 1), in accordance with ozone sonde^{28,29}, airborne^{3,25,30,31}, and satellite observations^{9,10,32} in recent years. The lowest chemical ozone losses are calculated for the winter 1996–97, that is, for a winter when unusual meteorological conditions suppressed the dynamical resupply of ozone, which resulted in very low ozone abundances in early 1997 (ref. 33). Previously observed chemical losses were most pronounced in the years 1992–93 and 1994–95 (see for example refs 9, 10, 28, 32) reaching values of ~50% in the lower stratosphere and strong ozone column reductions (Table 1). In 1992–93, the chemical ozone column loss is partly masked by a particularly strong descent in the vortex^{6,10}. Despite the severe chemical losses, the ozone columns (Table 1) and the vertical profiles^{10,28} of 1992–93 show therefore larger ozone abundances than were observed in 1995–96. During winter and spring of 1995–96 the Arctic vortex was both colder and more persistently cold than usual^{6,15}, leading to frequent PSC formation and subsequent chlorine activation of large vertical extent and uncharacteristically long duration (see Fig. 1 and ref. 21). We have shown here that the anomalously low ozone levels concurrently observed throughout the lower stratosphere^{6,24,27} are the result of unprecedented chemical ozone losses.

Despite the current phasing-out of chlorofluorocarbon production in the developed world, increasing stratospheric chlorine concentrations are still expected during the next few years¹⁸. Furthermore, even though there is a large interannual variability

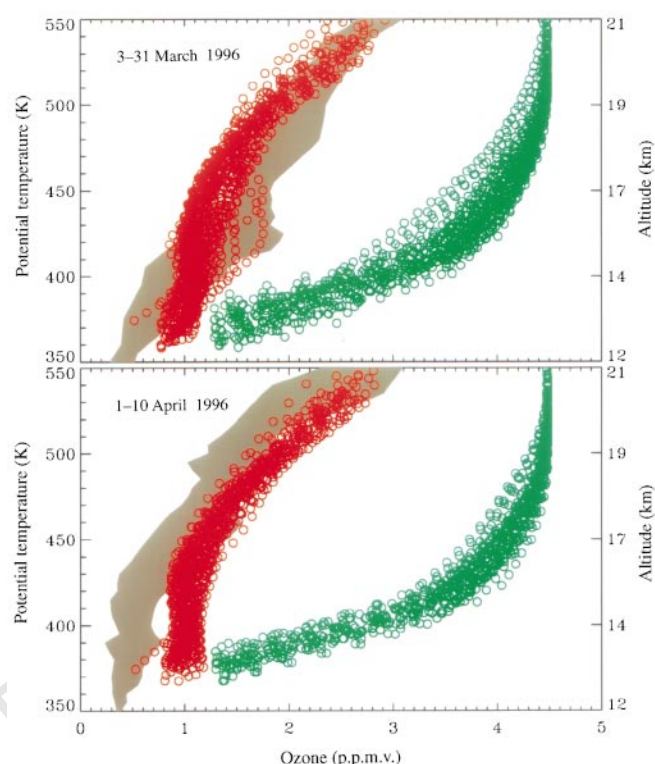


Figure 2 Vertical profiles of ozone mixing ratios (red symbols) measured by HALOE inside the polar vortex in March 1996 (top panel) and early April 1996 (bottom panel). Potential temperature Θ is used as the altitude scale to eliminate ozone variations caused by adiabatic, dynamical processes; approximate geometric height is shown on the right axis. All HALOE observations clearly inside the vortex in March and early April 1996 are shown. Also shown is the proxy ozone mixing ratio $\hat{O}_3$ expected if chemical processing had not occurred (green symbols) derived from the simultaneous methane measurements¹⁰. For comparison, the range of ozone mixing ratios measured in Antarctica in 1985 at the Georg Forster station (71°S) by ozone sondes²⁶ is shown for corresponding seasons: compare September (top panel, grey shaded area) with March in the Northern Hemisphere, and the first half of October (bottom panel, grey shaded area) with early April in the Northern Hemisphere.

Table 1 Calculated chemical loss in column ozone in the Arctic over six winters

Year	Period		Chemical ozone loss (DU)	Observed column (DU)
1991–92	29 March–2 April 1992	SR	104 (14)	268 (9)
1992–93	24 March–2 April 1993	SR	133 (14)	245 (10)
1993–94	17 March–2 April 1994	SR	102 (10)	230 (20)
1994–95	15–27 March 1995	SR	116 (20)	198 (11)
	31 March–12 April 1995	SS	81 (26)	212 (18)
1995–96	3–17 March 1996	SR	159 (19)	187 (11)
	26–31 March 1996	SS	121 (11)	192 (7)
	1–5 April 1996	SS	130 (9)	195 (5)
	6–15 April 1996	SS	131 (9)	210 (18)
1996–97	26–31 March 1997	SS	53 (23)	202 (15)

Calculated average ozone column losses in the altitude range 12–21 km (corresponding to 150–25 hPa; potential temperature, 350–550 K; CH₄, 1.5–0.5 p.p.m.v.). The difference between individual HALOE vertical ozone profiles inside the Arctic vortex and the proxy ozone (Fig. 2) is integrated to obtain an estimate of the chemical column loss¹⁰. The uncertainty in the derived ozone column loss due to the uncertainty in the proxy ozone (Fig. 1) is ~25 DU. Also listed is the ozone column above 100 hPa observed by HALOE. The time periods over which vortex observations are available differ from year to year owing to the HALOE viewing geometry and the varying shape of the vortex. Averages over data measured at sunrise (SR) and sunset (SS) are computed and, as an indication of the variation among the individual observations, the standard deviation is given in brackets. We note that the data in this table are derived from HALOE measurements processed with HALOE software version 18, in contrast to the values reported in ref. 10. There is a possibility of a negative bias of <10% in version 18 HALOE sunrise CH₄ data at low altitudes (below ~50 hPa) (L. L. Gordley, personal communication). Sensitivity calculations indicate that the maximum overestimate of the calculated change due to chemistry in the ozone column for sunrise observations is 35 DU.

in Arctic winter temperatures, there are indications of a cooling of the stratosphere as a result of increases in radiatively active trace gases^{16,17}. Should stratospheric temperatures as low as in 1996 occur while chlorine concentrations remain elevated, large ozone losses will recur. Studies of previous Arctic winters^{2,3,4,34} concluded that—had the stratospheric temperatures remained low in late winter, as in 1996^{6,15}—substantial ozone loss could have occurred, even for the lower chlorine loading present in 1989⁵. As stratospheric chlorine is projected to remain above those levels until after 2010⁵, the risk of extreme ozone losses in the Arctic will persist for more than a decade to come.

Model calculations (see, for example, ref. 35) indicate that pre-industrial chlorine levels were not sufficient to cause chemical ozone depletion of more than a few per cent. Since the 'ozone hole' was first reported in 1985^{13,14}, however, the intensity of annual ozone depletion over Antarctica has continued to increase substantially^{1,5,14}, in step with increasing stratospheric chlorine concentrations. The term 'ozone hole', coined in the mid-1980s³⁶, is used to describe large deviations of total ozone (~100 DU) from the long-term Antarctic mean, which are ultimately caused by anthropogenic release of stable halogen compounds into the atmosphere. In the light of this definition, the ozone losses observed during the boreal winter of 1995–96 might be considered to constitute the first clear occurrence of an 'Arctic ozone hole': although the observed drop in ozone column abundance was only ~50 DU, the calculated loss due to chemical depletion (and the deviation from mean climatological values) reached about 120–160 DU—a loss greater than observed in Antarctica in 1985. □

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The last two geomagnetic polarity reversals recorded in high-deposition-rate sediment drifts

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High-resolution records of geomagnetic polarity transitions are rare owing to the difficulty of preserving in the geological record details of a process with a duration of only a few thousand years. Lavas can record the instantaneous field on cooling, but lava records are themselves discontinuous and therefore polarity transition fields are seldom captured. Sedimentary records are more continuous, but typical deep-sea sedimentation rates of a few cm kyr⁻¹ are too low to record transition fields adequately. Here we report transition records from North Atlantic drift deposits with high sedimentation rates (~12 cm kyr⁻¹) at the Ocean Drilling Program sites 983 and 984. Virtual geomagnetic polar (VGP) paths are similar within and between sites for the last two geomagnetic reversals, attesting to a memory effect spanning back-to-back reversals. The complex VGP paths feature two VGP clusters (one located in northeastern Asia and the other in the South Atlantic Ocean off North America), and provide a link between the longitudinally constrained transitional VGPs often recorded in sediments^{1,2}—which represent highly smoothed renditions of transition fields—and the scattered transitional VGPs³ and VGP clusters⁴ observed in lava records.

Although many sedimentary polarity transition records have yielded VGP paths longitudinally constrained to the Americas or its antipode over eastern Asia^{1,2}, there is considerable scepticism as to whether this reflects the configuration of the geomagnetic field.

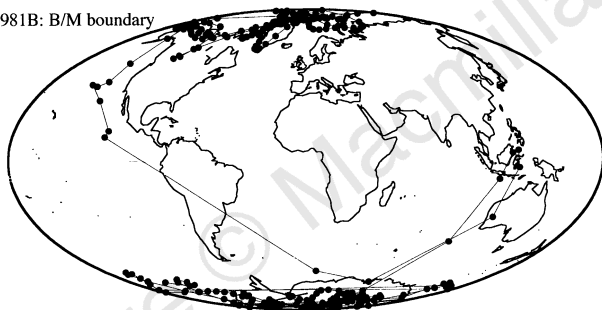
Alternative explanations include uneven site distribution and flattening of the magnetization vector during intervals of low geomagnetic intensity⁵⁻⁸. In recent years, the preferred antipodal VGP paths have been supported by improved site distribution^{9,10}, but accounting for them in terms of recurring transition field configurations has remained controversial. In view of the sedimentation rates of many of the records involved, the preferred VGP paths must be smoothed by the remanence acquisition process. This is borne out by the contrasting nature of sedimentary and lava records of polarity transitions. Longitudinally constrained VGP paths are not seen in the lava record where the transitional VGPs are either clustered in groups in the vicinity of Australia or off South America⁴, or randomly dispersed over the globe³.

One of the objectives of ODP Leg 162 was the acquisition of high-resolution climate records from the Feni drift (55.5° N, 14.7° W, Site 980/981), Gardar drift (60.4° N, 23.6° W, Site 983) and Bjorn drift (61.4° N, 24.1° W, Site 984). Based on magnetic polarity stratigraphy, the mean sedimentation rate across the Brunhes/Matuyama (B/M) boundary at Site 981 was $\sim 5.5 \text{ cm kyr}^{-1}$, similar to the B/M sedimentation rates at DSDP Site 609 which hitherto has yielded some of the most detailed polarity transition records¹¹. For sites 983 and 984, between the B/M boundary and the top of the Jaramillo subchron, the mean sedimentation rates are 12 and 11.5 cm kyr⁻¹, respectively; and within the Brunhes chron, the mean sedimentation rates are 11.6 and 13.8 cm kyr⁻¹, respectively.

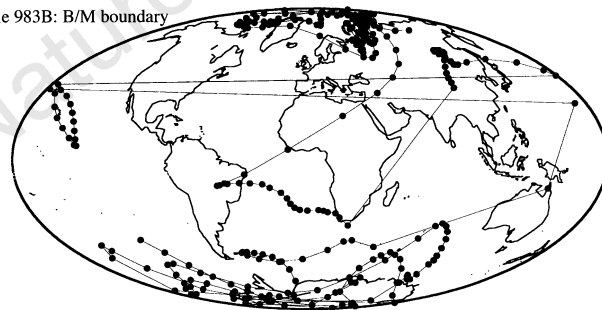
The B/M boundary was sampled in cores from two holes at Site 983, three holes at Site 984 and one hole at Site 981. Each 1.5-m core section close to the B/M boundary was sampled using U-channels. U-channels are typically 1.5 m in length with a $2 \times 2 \text{ cm}$ square cross-section and a clip-on lid constituting one of the sides¹². U-channel samples were measured using the high resolution small-access 2-G pass-through magnetometer¹³ at Gif-sur-Yvette (France). The measurements were all carried out at 1-cm intervals but the response function of the pick-up coils of the magnetometer has a width of $\sim 4 \text{ cm}$, so only every fourth measurement, at best, is independent.

Magnetic hysteresis data are consistent with magnetite being the only magnetic mineral present. Stepwise alternating field demagnetization of natural remanent magnetization was carried out at the following peak fields: 10, 15, 25, 30, 35, 40, 45 and 60 mT. Component magnetization directions were calculated at 1-cm intervals for the 25–60 mT demagnetization range using the standard least-squares method¹⁴ which yields a maximum angular deviation (MAD) value indicating the uncertainty in definition of the component. The demagnetization range (25–60 mT) for definition of the magnetization component was based on the demagnetization field ($\sim 20 \text{ mT}$) at which the steeply dipping drill string magnetization was removed. MAD values are larger in the vicinity of polarity reversals than elsewhere in the core, but at Site 983, for both the B/M boundary and the top Jaramillo reversal, MAD values

Hole 981B: B/M boundary



Hole 983B: B/M boundary



Hole 983C: B/M boundary

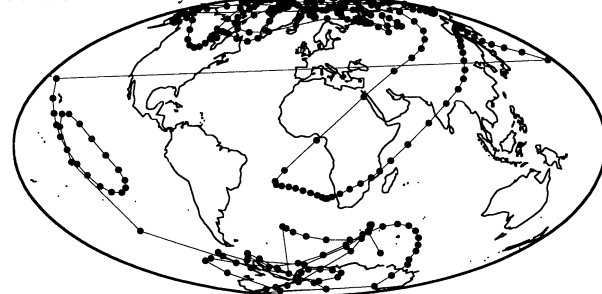
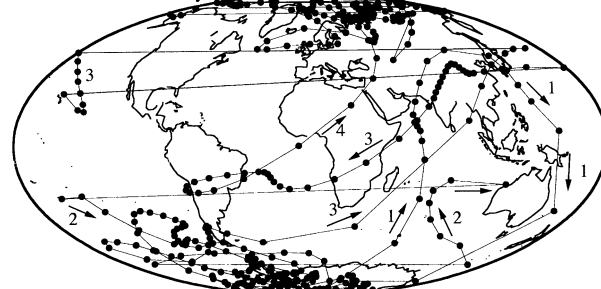
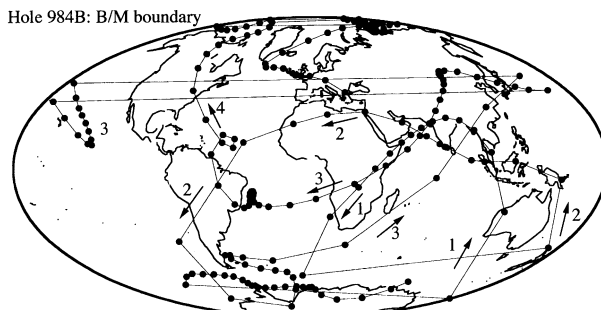


Figure 1 Virtual geomagnetic polar (VGP) paths for the reverse to normal polarity transition at the Brunhes/Matuyama boundary at Hole 981B, and two holes from Site 983. Sedimentation rates at Site 983 are about twice those at Site 981.

Hole 984A: B/M boundary



Hole 984B: B/M boundary



Hole 984C: B/M boundary

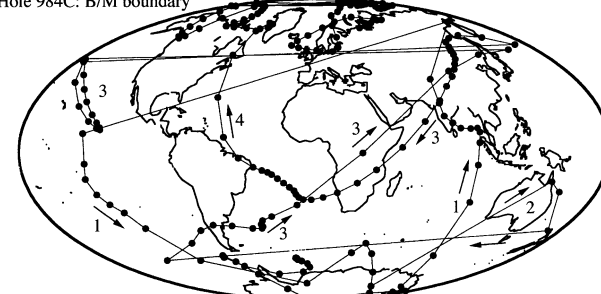


Figure 2 Virtual geomagnetic polar (VGP) paths for the reverse to normal polarity transition at the Brunhes/Matuyama boundary at three holes from Site 984.

are $<10^\circ$. At Site 984, for the B/M boundary, MAD values are larger ($<20^\circ$), reflecting less well defined magnetization components. Component declinations were orientated using data from the shipboard 'tensor multishot' core orientation tool, although, for Hole 984C, this tool was not used and orientation was performed by rotating mean declinations (outside the reversal) to north-south. Component directions, computed at 1-cm intervals across the polarity transitions, were used to compute virtual geomagnetic poles (VGPs).

At Hole 981B, B/M transitional VGPs track over the Americas (Fig. 1) reminiscent of the B/M boundary record at Site 609¹¹ and consistent with the longitudinally constrained VGP paths^{1,2}. Sites 983 and 984 have approximately double the sedimentation rate of Site 981, and therefore we expected more detailed records at these sites. At Site 983, the B/M VGP path has a Pacific clockwise loop followed by a northeast Asian cluster, then a South Atlantic cluster, and from there the VGPs move to high northerly latitudes (Fig. 1). At Site 984, where the estimated sedimentation rates are slightly greater than at Site 983, the VGP path takes on a new complexity (Fig. 2). The Pacific clockwise loop, and northeast Asian and South Atlantic clusters, are still present, as for Site 983, but the VGP path is augmented by two loops (marked loop 1 and loop 2 in Fig. 2) which predate the Pacific loop.

For the normal to reverse polarity transition at the top of the Jaramillo subchron at Site 983, the records from the three holes are

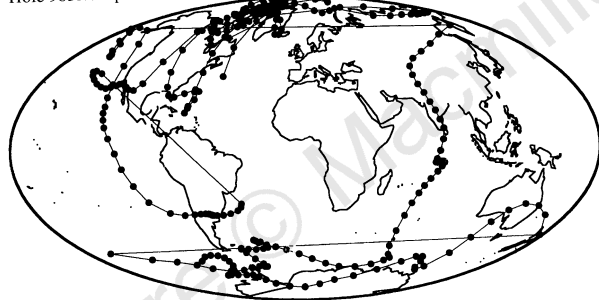
similar (Fig. 3). The VGP paths pass through the northeastern Pacific and the Americas to a South Atlantic cluster, then back to northerly latitudes to an apparent cluster in western North America, then through eastern Asia and the Indian Ocean to arrive at high southerly latitudes. A final loop over Australia is apparent before the reverse polarity state is established. The top Jaramillo record appears to have some of the same features as the B/M record but in reversed order. The Australian loop, northeast Asia cluster, and South American cluster appear in this order for the B/M reversal, and in the opposite order for the top Jaramillo reversal. This implies a memory spanning successive reversals, which cannot be attributed to fluid motions in the outer core, but rather to lateral heterogeneity in the lowermost mantle². The northeast Asian, South Atlantic (and North American) clusters coincide with fast P-wave propagation in the lower mantle and with near-radial downward flux centres associated with today's surface field stripped of its axial dipole⁴. It has been inferred from the palaeomagnetic record that such flux concentrations may be long-standing features of the geomagnetic field¹⁶ which may therefore influence successive polarity transitions.

No other sedimentary polarity transition records show similar clustering of VGPs, however, no other polarity transition data are available from sediments with comparable accumulation rates. Existing sedimentary polarity transition records are often similar to the record obtained at Hole 981B (Fig. 1) where the VGP path tracks over the Americas or eastern Asia. Longitudinal constraining of transitional VGP paths in sediments^{1,2} is therefore a result of the low resolution (low sedimentation rates) of the records and the smoothing effect of the remanence acquisition process. The South Atlantic VGP cluster, apparent in the Site 983 and Site 984 records, more or less coincides with the South Atlantic cluster compiled by Hoffman⁴ mainly from Plio-Pleistocene volcanic polarity transition data from the Hawaiian islands ($\sim 19^\circ$ N). Many, but not all, transitional VGP clusters from volcanic Southern Hemisphere sites lie in the vicinity of Australia. Examples include Plio-Pleistocene data from the Society Islands⁴ and the B/M record from Chile¹⁵. The Steens Mountain (42° N, 118° W) volcanic record of a Miocene reverse-to-normal polarity transition^{17,18} showed a similar oscillating or stop-go VGP pattern to that seen at sites 983 and 984, with poorly defined VGP clusters in the north Pacific and off South America.

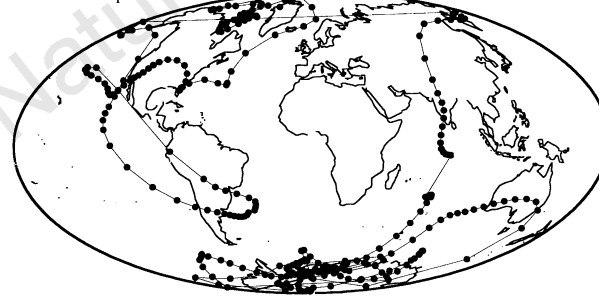
The prominent VGP loop in the Pacific for the B/M records at sites 983 and 984 (Figs 1 and 2) has clockwise looping. Other loops such as the precursor loops at site 984 (Fig. 2) do not have the same sense of looping. Although VGPs were computed every 1 cm from component magnetization directions, each point is not independent of the next (each fourth or fifth point is independent). Hence, the VGPs joining the VGP clusters in these records may be artefacts of smoothing not only by the remanence acquisition process but also by the measurement procedure. We suspect that the VGP jump from the northeast Asian cluster to the South Atlantic is abrupt, and that the precursor loops at Site 984 and VGPs between clusters may be smoothing artefacts. This is borne out by the differences in the nature of loop 1 and loop 2 at the three holes at Site 984 (Fig. 2). Differences among the B/M transition records at sites 983 and 984 are attributed to the vagaries of this imperfect recording medium. We note that the Pacific loop is consistent among the records (Figs 1 and 2) and therefore is probably indicative of westward drift of secular variation centres according to Runcorn's rule¹⁹ and numerical simulations²⁰.

For the $\alpha\omega$ class of geodynamos²¹, it has been demonstrated that the large-scale shear that causes the ω effect is symmetric about the Equator, whereas the α effect is antisymmetric with respect to the Equator. This has led to a subdivision of the geodynamo into two non-interacting components made up two distinct families of spherical harmonics²². The so-called antisymmetric family comprises the axial dipole (g_1^0), the non-axial quadrupoles and certain higher order octupole terms; and the symmetric family comprises

Hole 983A: top Jaramillo



Hole 983B: top Jaramillo



Hole 983C: top Jaramillo

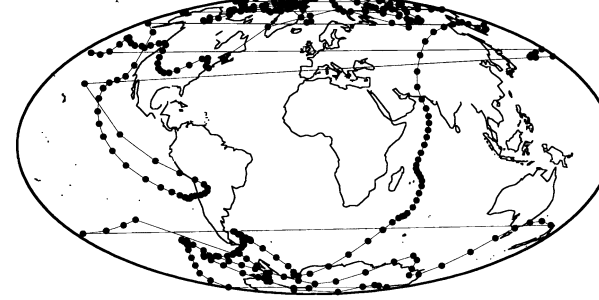


Figure 3 Virtual geomagnetic polar (VGP) paths for the normal to reverse polarity transition at the top of the Jaramillo subchron at three holes from Site 983.

the inclined (equatorial) dipoles, the axial quadrupole and other higher-order terms²³. This subdivision has a physical meaning in terms of the geodynamo, and is therefore preferable to treating the spherical harmonics individually or grouped by degree. With this in mind, Hoffman²⁴ tested the VGP clusters and their site distribution for equatorial symmetry. The North Atlantic sediment drifts (60–61° N) yield a South Atlantic cluster and a northeast Asian cluster for the B/M boundary. The South Atlantic VGP cluster in volcanic polarity transition data emanates largely from Hawaii (~19° N), and an Australian VGP cluster emanates mainly from the Southern Hemisphere, the Society Islands (~18° S) and Chile (36° S). As the Australian and northeast Asian clusters, and the North Atlantic and Chilean sites, are located approximately on a common meridian but different hemispheres, the transitional fields at the B/M boundary appear to have been dominated by the equatorially symmetric family of spherical harmonics which includes g_1^1 , h_1^1 (equatorial dipoles) and g_2^0 (axial quadrupole).

Models of polarity transition fields have been stymied by the apparent inconsistency of longitudinally constrained VGP paths^{1,2} from sediments and random VGPs³ or VGP clusters^{4,24} recognized in the volcanic data. The presence of VGP clusters in transition records from these drift sediments goes some way towards resolving this inconsistency, indicating that longitudinally constrained sediment transition records are highly smoothed renditions of transition fields. □

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El Niño Southern Oscillation and tuna in the western Pacific

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Nearly 70% of the world's annual tuna harvest, currently 3.2 million tonnes, comes from the Pacific Ocean. Skipjack tuna (*Katsuwonus pelamis*) dominate the catch. Although skipjack are distributed in the surface mixed layer throughout the equatorial and subtropical Pacific, catches are highest in the western equatorial Pacific warm pool, a region characterized by low primary productivity rates¹ that has the warmest surface waters of the world's oceans (Fig. 1). Assessments of tuna stocks indicate that recent western Pacific skipjack catches approaching one million tonnes annually are sustainable². The warm pool, which is fundamental to the El Niño Southern Oscillation (ENSO) and the Earth's climate in general^{3–5}, must therefore also provide a habitat capable of supporting this highly productive tuna population. Here we show that apparent spatial shifts in the skipjack population are linked to large zonal displacements of the warm pool that occur during ENSO events^{5,6}. This relationship can be used to predict (several months in advance) the region of highest skipjack abundance, within a fishing ground extending over 6,000 km along the Equator.

There is a permanent convergence of surface-layer water masses on the eastern edge of the western Pacific warm pool⁵. The convergence zone is identified by a well-marked salinity front⁵, *in situ* observations of currents⁷ and nutrients⁸, and simulated trajectories of drifters⁵. It is induced by westward advection of cold, saline water from the central-eastern equatorial Pacific encountering a sporadic eastward advection of warm, low-salinity water from the western equatorial Pacific, generated by westerly wind bursts. Spectacular zonal displacements of the convergence zone over 50° of longitude occur in phase with the warm and cold phases of the ENSO cycle, with eastward displacement occurring during El Niño episodes and westward displacement occurring during La Niña episodes. Convergence zones and fronts are important aggregating mechanisms of plankton and micronekton^{9–13}, and therefore also of larger predators such as tuna^{14,15}. We would expect, therefore, that the distribution of skipjack tuna would respond to displacement of the convergence zone, and that this might provide a basis for the prediction of good fishing grounds. We tested this hypothesis by correlating observations of skipjack relative abundance with a proxy for location of the convergence zone, the 29 °C sea surface temperature (SST) isotherm, and with the southern oscillation index (SOI). A proxy variable for the location of the convergence was required because the spatio-temporal coverage of salinity, nutrient and surface current observations was insufficient to map the convergence location in a continuous time series suitable for study. Movement of the 29 °C SST isotherm is highly correlated with both the movement of the salinity front and movement of the convergence zone determined by drifter trajectories⁵. This isotherm therefore seems to be a reasonable proxy for the convergence.

Catch per unit effort (CPUE) data from commercial fisheries are often used to measure the relative abundance of fish stocks¹⁶. Average global CPUE for the United States purse seine fleet (see Methods) shows that the skipjack fishing ground extends across 60° of longitude (Fig. 2a), with strong interannual variations in location (Fig. 2b). The purse seine fleet was concentrated west of 160° E during the well-defined La Niña period of 1988 to 1989, the short

intermediate period in late 1990 to early 1991, and the 1995 La Niña. During El Niño years (1992–1994), the fleet extended its activities as far east as 160°W. To calculate the correlation between CPUE and environmental indices, we computed G , a longitudinal gravity centre of CPUE. G is correlated ($R = 0.75$, zero lag) with the 29°C SST isotherm (Fig. 2b), and the amplitude of the variables is very similar (standard deviation ratio of 0.8). Despite a generally good relationship, at times (early 1991, early 1992, early 1995) G is located a long way to the west of the 29°C isotherm. Such departures from the relationship could suggest some inconsistency in the identification of the convergence by the 29°C isotherm, variability in the response of the skipjack population to movement of the convergence, imperfect location of highest skipjack abundance by the purse seine fleet, or a combination of these factors. Nevertheless, it seems that a strong relationship exists between the location of the convergence and skipjack relative abundance.

Shifts in G of up to 40° of longitude (nearly 4,000 km) during periods as brief as six months occurred on several occasions (Fig. 2b). The magnitude and speed of these shifts are not inconsistent with the mobility of individual skipjack. The South Pacific Commission's skipjack-tagging database has numerous records of tagged skipjack showing displacements of this order (South Pacific Commission, unpublished data). Also, during several periods of rapidly changing G , shown in Fig. 2b, the magnitude and direction of observed movements of tagged skipjack are consistent with the changes in G . During mid 1991 to early 1992, observed long-distance (>10° longitude) movements of tagged skipjack were predominantly towards the east (Fig. 3a, b), consistent with the shifts in CPUE and the 29°C isotherm (Fig. 2b). In contrast, during March to October 1992, westerly movement of tagged skipjack predominated (Fig. 3c), again consistent with CPUE and 29°C isotherm shifts (Fig. 2b). These data suggest that changes in skipjack CPUE occurring in phase with changes in the location of the

convergence at the eastern edge of the warm pool are likely to be due to movement of skipjack.

The evolution of the SOI, which characterizes the atmospheric component of ENSO¹⁷, precedes the zonal displacement of the 29°C SST isotherm ($R = -0.89$) and the CPUE gravity centre ($R = -0.75$) by two months (Fig. 2c, d). This evidence of ENSO-related zonal displacement of the skipjack distribution could allow the prediction of favourable fishing grounds two months in advance, according to the results of the cross-correlation. Furthermore, indices of ENSO, such as SST variations in the equatorial band and the SOI, can be predicted by statistical models and by dynamically coupled ocean–atmosphere models up to one year in advance^{18–20}. Oceanic general circulation models are also able to predict the salinity front at the eastern edge of the

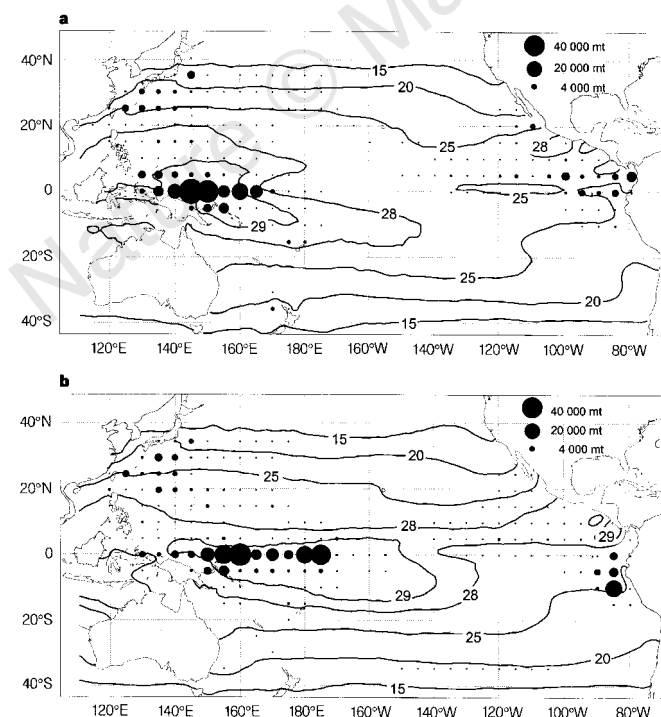


Figure 1 Distribution of skipjack tuna catch (tonnes) and mean sea surface temperature (SST, in °C) in the Pacific Ocean. **a**, In the first half of 1989 (La Niña period). **b**, In the first half of 1992 (El Niño period). The effect of ENSO on the location of the warm pool (SST > 28–29°C) and the distribution of skipjack catch is clearly evident.

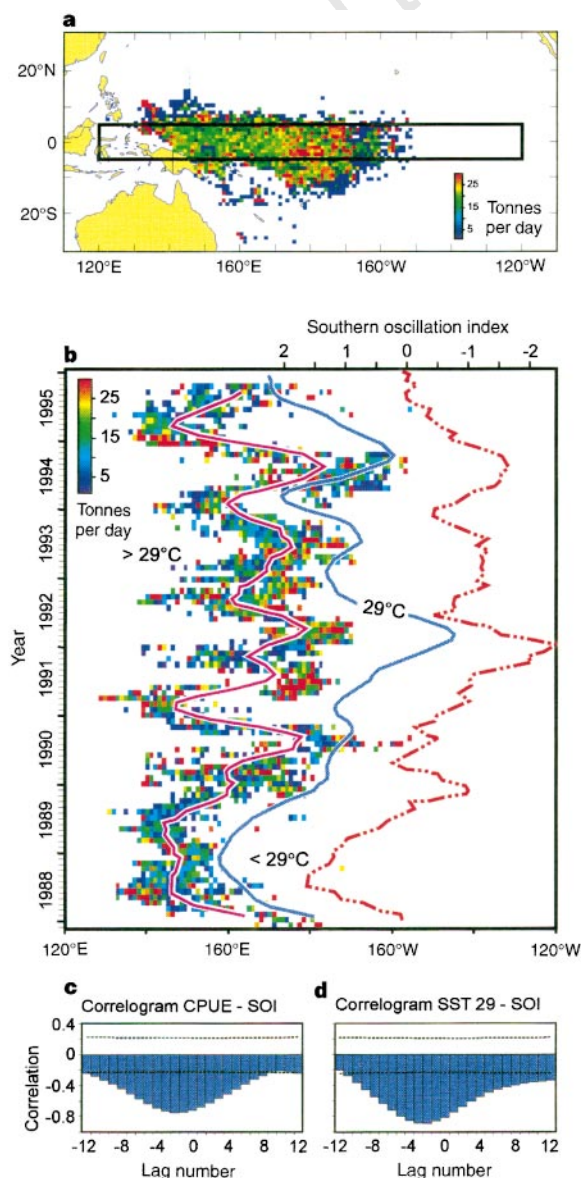


Figure 2 Skipjack tuna CPUE of the United States purse seine fleet in the western equatorial Pacific. **a**, Average CPUE for 1988 to 1995. The rectangle delimits the time–longitude section (**b**) of monthly mean CPUE; the pink line is the longitudinal gravity centre of CPUE (G), the blue line is the 29°C sea surface temperature (SST) isotherm, and the broken red line is the southern oscillation index (SOI); each variable was smoothed with a five-month moving average. Correlation coefficients between G and the SOI (**c**), and between the 29°C SST isotherm and the SOI (**d**), are estimated by the cross-correlation function (lags in months; dotted lines represent coefficients significant at $P = 0.05$).

warm pool with good accuracy⁵, and could improve the forecast in the location of the convergence. The lead time of prediction of favourable fishing grounds could therefore probably be increased by the use of these models. Such forecasts could result in improved catches and considerable reductions in searching time and costs for the fishing fleet.

The warm pool is characterized by low primary productivity (coastal waters excepted), which contrasts strongly with the enriched water masses of the adjacent central and eastern Pacific equatorial upwelling^{1,21}. Given the very high energetic requirements of skipjack²², and the short trophic linkage between their diet of epipelagic zooplankton and micronekton^{23–25} and the primary productivity of the surface layer, it is initially counter-intuitive that the world's largest tuna stock occurs in the oligotrophic western Pacific warm pool. Our results indicate that the presence of a large front of oceanic convergence is probably important for maintaining the skipjack stock in the warm pool. However, mechanisms that provide the necessary biomass of forage for the skipjack tuna stock have not yet been identified. A plausible explanation could involve the zonal transport of planktonic communities originating in the Pacific equatorial upwelling by the westwards-flowing south equatorial current. Such zonal equatorial advection can produce westward displacement of planktonic communities of 1,800–2,500 km (refs 26, 27). During this westward transport, the planktonic community would mature for up to several months, consistent with the residence time of 3–4 months of drifting buoys in the 5° N–5° S equatorial divergence zone²⁷. Westward transport would terminate on exiting the westward flow, through lateral drift or encoun-

tering the convergence zone at the eastern edge of the warm pool. The increasing mobility of the organisms through the upper trophic levels²⁸ and the large ENSO-related zonal movements of the convergence zone are likely to increase the patchiness of the spatial distribution of the secondary and tertiary production in a large zonal band associated with the physical front. The location of high abundance of skipjack tuna, centred on the longitudinal gravity centre of CPUE and extending about 14° in longitude ($\pm$ one standard deviation), may reflect this zone of enrichment in secondary and tertiary production. Unfortunately, direct observation of zooplankton and micronekton distributions on a spatial and temporal scale that would be required to test the mechanism described above remains problematic. Recently developed physical–biological coupled models²⁹ (P.L., unpublished data) are a promising means of investigating potential causal mechanisms associated with the strong relationship we have observed between skipjack tuna abundance, the water mass convergence at the eastern edge of the warm pool, and their drastic zonal displacements associated with ENSO. Because the signal is strong and the correlation high, the relationship could be a useful test of these models. The prediction of ENSO and, as a consequence, the location of the highest abundance of skipjack tuna, has important implications for the commercial tuna fishing industry. It may also allow fisheries management authorities to distinguish between changes in local fish abundance caused by movement to other areas, and changes resulting from the impacts of fishing. □

Methods

Gravity centre of CPUE. CPUE as an index of fish stock abundance will be most reliable when the sampling units (fishing vessels) are as homogeneous in their characteristics and operating behaviour as possible¹⁶. We used only United States purse seine CPUE data because: this highly mobile fleet consists of similar vessels using similar fishing techniques; unlike other purse seine fleets, this fleet has had virtually unrestricted access since 1988 to the exclusive economic zones of Pacific Island countries under the South Pacific Tuna Treaty, as well as adjacent high seas; and catch and effort data quality and coverage for log books submitted by the fleet is high. The time–longitude section of monthly skipjack catch (in tonnes) and effort (in days fishing) was produced by aggregating the data in the area 5° N–5° S, 120° E–120° W by months and 1° of longitude. The longitudinal gravity centre of CPUE in month j (G_j) is then defined as: $G_j = \sum_i L_i (C_{ij}/E_{ij}) / \sum_i (C_{ij}/E_{ij})$, where L_i denotes the longitudinal mid-point of the i th area of 1° longitude width between 5° N and 5° S, C_{ij} is the skipjack catch in area i in month j , and E_{ij} is the number of fishing days in area i in month j .

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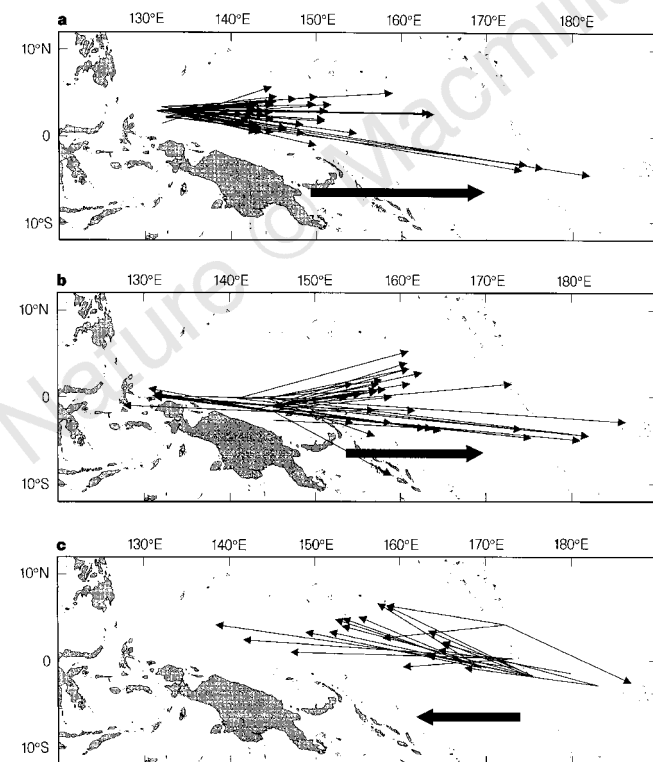


Figure 3 Displacements of tagged skipjack tuna. **a**, Tuna released April 1991, recaptured before February 1992. **b**, Tuna released May 1991, recaptured before February 1992. **c**, Tuna released March 1992, recaptured before October 1992. For clarity, only tagged fish recaptured in the zone 10° N–10° S and more than 10° of longitude east or west of their release location are plotted. Tagging data were compiled from records of a large-scale tagging programme carried out by the South Pacific Commission during 1990 to 1992 (ref. 30). Thick arrows indicate the direction and magnitude of displacement of the skipjack CPUE gravity centre (see Methods) during the tag recapture periods.

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Radical alterations in the roles of homeobox genes during echinoderm evolution

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Echinoderms possess one of the most highly derived body architectures of all metazoan phyla, with radial symmetry, a calcitic endoskeleton, and a water vascular system^{1,2}. How these dramatic morphological changes evolved has been the subject of extensive speculation and debate^{3–5}, but remains unresolved. Because echinoderms are closely related to chordates and postdate the protostome/deuterostome divergence^{2,3,6,7}, they must have evolved from bilaterally symmetrical ancestors^{1–6}. Here we report the expression domains in echinoderms of three important developmental regulatory genes (*distal-less*, *engrailed* and *orthodenticle*), all of which encode transcription factors that contain a homeodomain⁸. Our findings show that the reorganization of body architecture involved extensive changes in the deployment and roles of homeobox genes. These changes include modifications in the symmetry of expression domains and the evolution of several new developmental roles, as well as the loss of roles conserved between arthropods and chordates. Some of these modifications seem to have evolved very early in the history of echinoderms, whereas others probably evolved during the subsequent diversification of adult and larval morphology. These results demonstrate the evolutionary lability of regulatory genes that are widely viewed as conservative.

Orthologues of the developmental regulatory genes *distal-less*, *engrailed* and *orthodenticle* are present in arthropods and chordates,

where there are similarities in their roles and expression domains: *distal-less* in proximodistal patterning during limb outgrowth⁹; *engrailed* in neurogenesis along the axis of the central nervous system¹⁰; and *orthodenticle* in the specification of anterior structures¹¹ (Fig. 1a). Each gene also has other functions during development^{8–11}, however, and it is not even clear whether these superficially similar roles are conserved between phyla or evolved independently^{12,13}. We examined the expression of these genes in several echinoderm species representing four of the five extant classes and a diversity of adult and larval morphologies within the phylum.

The three homeoproteins are expressed in fivefold radially symmetrical patterns during development of the radial adult body (Fig. 2) in all echinoderms examined (Figs 3d,e, 4c–e, 5). Based on time of expression, none of these genes seems to be involved in establishing radial symmetry, but all three act downstream of the genes that are. Most radial expression domains correspond to structures that are unique to echinoderms, such as podia, endoskeleton, spines, arm-tip ectoderm, and imaginal rudiment. Podia (tube-feet) function in locomotion, feeding and sensory perception^{1,2}, and are extensions of the water vascular system, an organ system unique to echinoderms^{1,2,6}. The gene *orthodenticle* is expressed in the podia of brittle stars (Fig. 4d) and sea urchins, and in the homologous¹⁴ buccal tentacles of sea cucumbers (sea stars were not examined). The gene *distal-less* is expressed in the podia of sea urchins⁹ (Fig. 3d) and sea stars, and in the buccal tentacles of sea cucumbers (Fig. 3e), but was not detected in the podia of brittle stars. In sea urchins, podial expression of *distal-less* begins within the imaginal rudiment at the distal end of the five primary podia soon after they form, and persists through early juvenile development. The calcitic endoskeleton of echinoderms evolved after their divergence from other deuterostome phyla^{2,4–6}. Ectodermal expression of *engrailed* in brittle stars (Fig. 5a–e) is restricted to the nuclei of cells that overlie boundaries between newly forming skeletal ossicles. This spatial correlation is present beginning with the synthesis of the first ossicles of the adult skeleton (Fig. 5a, b) and persists as far into postmetamorphic juvenile development as we examined (Fig. 5c–e). This association suggests that *engrailed* is involved in patterning axial skeletogenesis, which is consistent with experimental evidence that skeletogenic mesenchyme cells in sea-urchin larvae rely in part on ectodermally derived cues for patterning¹⁵. The spines of sea urchins are specialized derivatives of the endoskeleton^{2,14}. Ectodermal expression of *distal-less* is evident at the tips of spines⁹, but was not detected in association with other skeletal elements in sea urchins or in other echinoderms examined. In brittle stars, *orthodenticle* is expressed in the ectoderm overlying the terminal ossicles at the tips of the arms, with diminishing expression proximally (Fig. 4e). Ectodermal expression was not detected overlying the homologous¹⁴ ocular ossicles in sea urchins. In sea urchins, a large imaginal rudiment gives rise to much of the postmetamorphic juvenile^{14,16}. In most sea urchins, an ectodermal invagination encloses the rudiment¹⁶. Initially, *distal-less* is expressed in all these ectodermal cells, but is later restricted to the distal (inner) end of the invagination (Fig. 3a–c). No *distal-less* expression was detected in the rudiment ectoderm of echinoderms from other classes.

Each of these cases probably represents recruitment (co-option) of a homeobox gene to a new developmental role^{17,18}, as the structures where expression occurs evolved after the divergence of echinoderms and chordates^{2,4–6}. Role recruitment implies that the downstream targets of these genes in echinoderms include loci that are different from those in other phyla. Both *engrailed* and *orthodenticle* exist as single-copy genes in sea urchins^{19,20}, and presumably other echinoderms, implying that these recruitment events occurred without gene duplication. (The copy number of *distal-less* has not been determined in any echinoderm.) In contrast, recruitment of developmental roles in chordates is often associated

with gene duplication followed by divergence of developmental roles^{17,21}. Some features of expression are probably homologous among echinoderm classes (such as *orthodenticle* expression in podia), implying an origin over 450 Myr ago²², whereas others have a more restricted phylogenetic distribution (such as *distal-less* in sea-urchin rudiments) and apparently evolved later (Fig. 1b).

Not all expression domains in echinoderms imply recruited developmental roles. In the brittle star *Amphipholis squamata* (Fig. 5f), *engrailed* is expressed in a subset of neuronal cell bodies (C. Lowe and M. Thorndyke, unpublished data) in paired ganglia along the five radial nerves. This expression is superficially similar to that in bilateral animals, in which *engrailed* is expressed within a

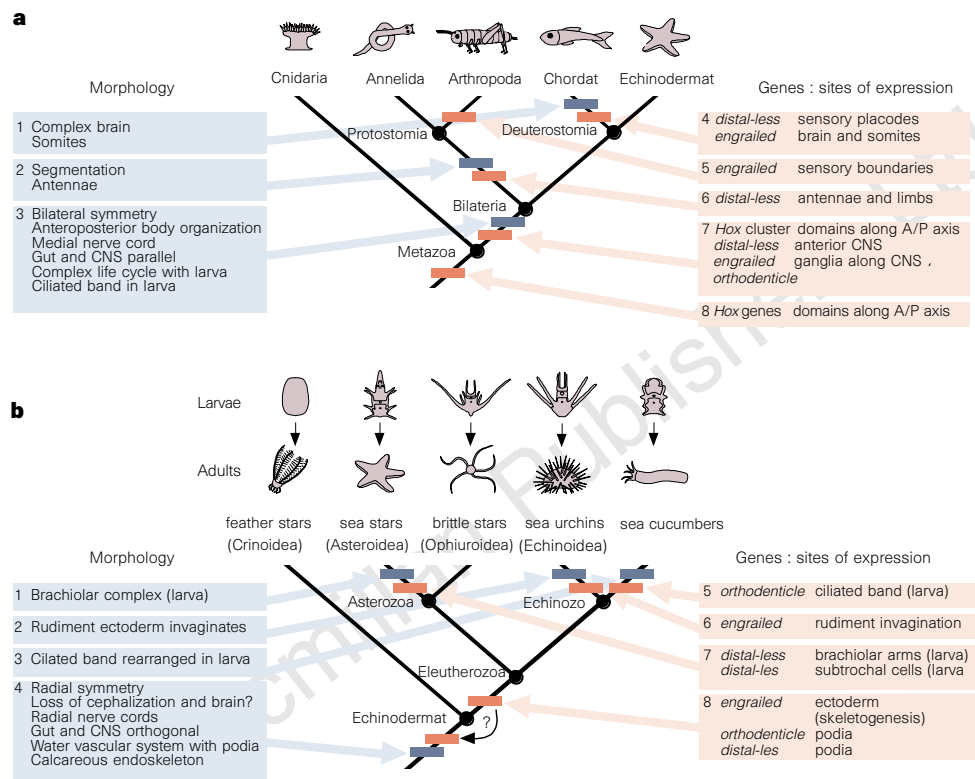


Figure 1 Evolutionary history of body architecture and regulatory genes within the Metazoa (**a**) and Echinodermata (**b**). Reconstructed changes in morphology (left) and in the developmental roles and expression domains of regulatory genes (right) are shown. The expression domains and roles of several regulatory genes are similar in arthropods and chordates (**a**, 8)⁸⁻¹¹, despite their phylogenetic disjunction and morphological disparity^{2,5,6}. These similarities probably reflect homology in basic architectural features, such as bilateral symmetry and an anteroposterior axis (**a**, 3) between protostomes and deuterostomes. Morphological features that distinguish phyla (such as **a**, 1) and groups of related phyla (**a**, 2) evolved later. In some such cases, changes in the developmental roles of regulatory genes (**a**, 5 and 7) have been identified that may have been involved in the origin of these derived structures^{9,10,17}. The origin of echinoderms followed the divergence of arthropods and chordates, and involved substantive modifica-

tions in basic body architecture (**b**, 2)¹⁻⁶; already present was a complex life cycle involving a larva that fed using a ciliated band (**a**, 3)¹⁻⁶. Data reported here and taken from the literature^{8-11,19} allow us to reconstruct changes in the expression domains of three regulatory genes during the origin and radiation of echinoderms. Several derived features of gene expression are shared by asterozoan and echinozoan echinoderm species (**b**, 7), implying that they were present before the divergence of these clades over 490 Myr ago²². The origin of these features could be even earlier (arrow near base of cladogram), but expression data are currently unavailable for crinoids, the most basal clade of extant echinoderms. Other features of gene expression appear to be limited to a single class (such as **b**, 4 and 6), suggesting later origins or subsequent losses within other lineages. Phylogenetic relationships are from refs 2, 6, 7 and 14.

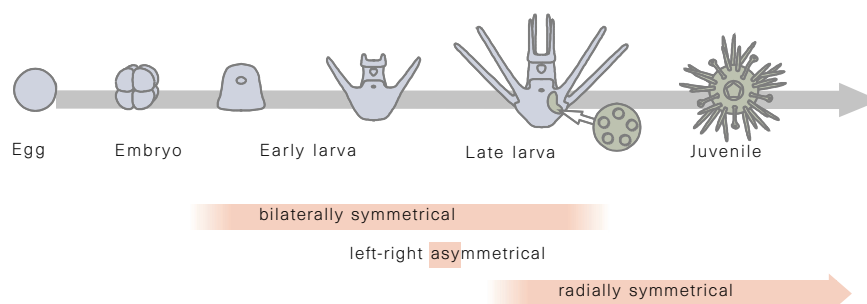


Figure 2 Summary of echinoderm development. Echinoderm embryos and larvae are bilaterally symmetrical^{16,16}. In late larvae, discrete populations of ectodermal and mesodermal cells on the left side of the body proliferate to form the imaginal adult rudiment (green crescent). The first radially symmetrical structures appear within the rudiment (disc near late larva). During metamorphosis, many

larval tissues (blue) are lost, and the rudiment gives rise to much of the juvenile (green). Domains of regulatory gene expression fall into three distinct symmetry classes: bilateral in embryos and early larvae; left-right asymmetric in late larvae, during establishment of the imaginal rudiment; and radial during and after metamorphosis.

serially repeated subset of ganglionic neurons along the antero-posterior axis^{10,23}. It is possible that a neurogenic role for *engrailed* is widely conserved among triploblastic animals. However, the radial nerves of echinoderms are ancestrally organized in a zigzag along the proximodistal axis of the arms, and only in sea stars and brittle stars are the ganglia arranged in bilateral pairs¹⁴. Thus the superficial similarity of *engrailed* expression between brittle stars and bilateral animals is probably convergent. Another possibility for a conserved role in echinoderms is that of *orthodenticle* in the ectoderm of sea-urchin embryos¹⁹.

Echinoderms begin development as bilaterally symmetrical embryos and larvae^{1,16}. A few bilaterally symmetrical gene expression domains are present in larvae, notably the ciliated band of a sea-cucumber larvae, the brachiolar arms of starfish larvae, and the mesenchyme cells of sea-star larvae. Ciliated bands are present in larvae from all five extant classes^{6,16}, and are used for locomotion and feeding⁶. The gene *orthodenticle* is expressed in ectodermal cells of the circumferential ciliated bands of a bilateral sea-cucumber larva (Fig. 4f), but was not detected in the ciliated bands of any other echinoderm larvae. This restricted phylogenetic distribution suggests role recruitment after the evolutionary origin of the ciliated band (Fig. 1b), as the ciliated band almost certainly pre-dates the origin of echinoderms^{2,6} (Fig. 1a). A similar case is the role of *even-skipped* in insect segmentation, which evolved after segmental body organization¹⁰. Brachiolar arms are used for attachment to the benthos during settlement and metamorphosis^{1,16}, and are present only in the larvae of sea stars¹⁶. Scattered cells within the ectoderm of

the brachiolar arms express *distal-less* (Fig. 3h), and, judging by their shape, number and position, they are probably neurons or neuronal precursors. Expression of *distal-less* was also detected in mesenchyme cells (Fig. 3f) lining the tips of the larval arms in sea stars. On the basis of their location, these appear to be subtrochal cells, which link muscles to the body wall in sea star larvae²⁴.

Each of these cases probably represents a role recruitment that occurred well after the origin of echinoderms, given that each is restricted to a single class. To our knowledge, these cases are the first reported examples of role recruitment associated with the evolution of exclusively larval structures. Many phyla differ from arthropods and chordates in patterning their larval and adult bodies at different times during development, and in containing larvae that are morphologically quite distinct from adults^{2,5,6,16}. Thus we predict that role recruitment associated with the origin and modification of larval structures will prove common in other metazoan phyla with complex life cycles, such as annelids, molluscs, nemerteans, platyhelminths and phoronids.

The paucity of conserved expression domains, and by implication developmental roles, for the three genes we examined contrasts with most comparisons between arthropods and chordates, which have emphasized evolutionary conservation^{8,11,25,26}. It seems reasonable that conservation in expression domains is tied to morphological conservation, and that altered expression domains may have produced many large-scale evolutionary changes in morphology^{5,10,17}. Accordingly, we predict that analyses of metazoan taxa with divergent adult body architectures (such as cnidarians, ctenophores,

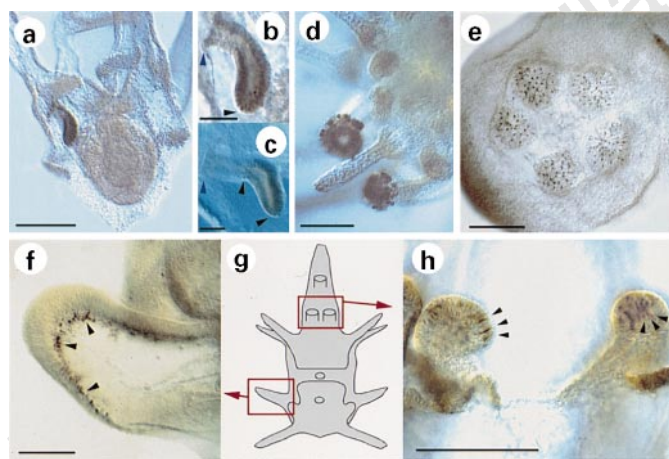


Figure 3 Expression of Distal-less protein. **a**, Mid-larva of the sea urchin *Strongylocentrotus droebachiensis*. Expression is present throughout the invagination that becomes the ectodermal component of the imaginal rudiment, which gives rise to the bulk of the adult body. This expression domain is present only on the left side of the larva. **b**, Close-up **a**, showing nuclear expression in all invaginating cells. In **b** and **c**, blue arrowheads mark the opening of the invagination, and black arrowheads mark the extent of *distal-less* expression. **c**, Close-up of imaginal rudiment from later larva, showing expression restricted to cells at the distal (innermost) end of the invagination. **d**, Juvenile sea urchin, *S. droebachiensis*. Expression is limited to the distal ends of podia and spines. **e**, Rudiment in mid-larva of the sea cucumber *Cucumaria miniata*. Scattered nuclei in the ectoderm express Distal-less protein in the five primary podia; these are probably neurons. **f**, Single arm from larva of a sea star (*Evasterias troschelii*) showing expression in mesodermal cells (probably subtrochal cells) lying just under the ectoderm near the arm tip (arrowheads). Expression of *distal-less* was not detected in other mesodermal cell types in asteroid larvae. **g**, Generalized late starfish larva showing the location of structures shown in **f** and **h**. **h**, Two brachiolar arms from the larva of *E. troschelii*. The brachiolar complex is used for attachment to the benthos during settlement¹. Scattered nuclei throughout the ectoderm of the distal pad in each brachiolar arm express Distal-less (arrowheads); these cells appear to be neurons. Scale bars: **a**, **d**–**g**, **i**, 100 μ m; **b**, **c**, 30 μ m.

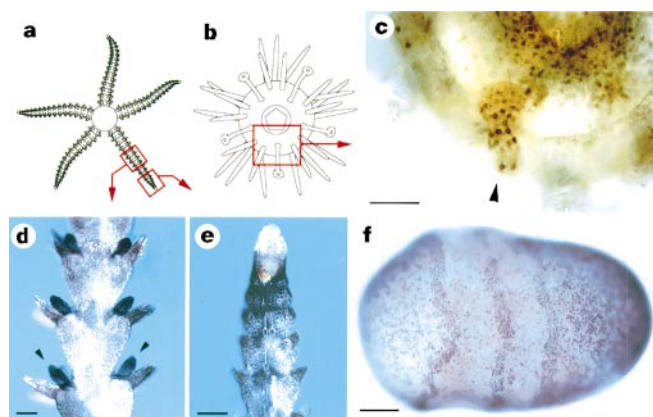


Figure 4 Expression of Orthodenticle protein. **a**, Generalized juvenile brittle star (oral view), showing location of structures shown in **d** and **e**. **b**, Generalized juvenile sea urchin (oral view), showing location of structures shown in **c**. **c**, Close-up of single podium (arrowhead) from the sea urchin *Strongylocentrotus droebachiensis*. Distal-less expression is restricted to nuclei along the length of the podium and surrounding the mouth. **d**, Oral surface of part of an arm from a juvenile brittle star (*Amphipholis squamata*). Ectodermal expression is evident throughout podia (arrowheads), which in brittle stars lack suckers. **e**, Tip of one arm from *A. squamata*. Expression is present at high levels throughout the ectoderm near the distal end of the arm, and declines proximally. **f**, Mid-larva of the sea cucumber *Psolus chitonoides*. Expression is evident in three circumferential stripes (4–6 cells wide) within the ectoderm. These cells bear large cilia and make up the ciliated band, the locomotory organ in echinoderm larvae. Most ectodermal cells between the circumferential bands lack cilia at this stage, but a few have not yet lost them; these cells probably correspond to the nuclei expressing *distal-less* protein that are scattered throughout the ectoderm. Scale bars: **c**, **f**, 100 μ m; **d**, **e**, 500 μ m.

brachiopods, bryozoans, rotifers and pterobranch hemichordates), will reveal many additional evolutionary changes in the developmental roles of homeobox genes that are widely considered conservative.

The highly derived body architecture of echinoderms evolved at least in part through extensive modifications in the roles and expression domains of regulatory genes inherited from their bilateral ancestors. Even the limited number of genes and species we examined demonstrates a remarkable evolutionary flexibility in genes that have previously been considered interesting mainly for their conserved roles in arthropods and chordates^{6,11,25,26}. However, comparisons between these phyla must be supplemented with data from additional phyla if we are to understand the role played by regulatory genes in producing morphological change^{5,25}. □

Methods

Adults and embryos. Adult echinoderms were collected intertidally or by Scuba from various sites around San Juan Island, WA, USA. Embryos, larvae and juveniles were reared in filtered sea water as described²⁷. Planktotrophic larvae were fed *Rhodomonas lens* and *Dunaliella tertiolecta*.

Antibody probes. Monoclonal antibody 4D9 (ref. 28) was used to detect Engrailed protein in the brittle star *Amphipholis squamata*. To authenticate binding, a fragment of *engrailed* spanning the epitope²⁸ was amplified from genomic DNA using primers 5'-CGCCCTCGCACAGCCTTCA-3' and 5'-TGGTTGTAMARDCCCTGHGCCAT-3', cloned into pCRScript (Stratagene), and sequenced (Sequenase, Amersham). The resulting sequence (Genbank accession no. U80674) was used to predict the amino-acid sequence of the

epitope. Artificial peptides were synthesized (Bio-Synthesis) to this region of Engrailed protein from *Drosophila melanogaster* (positive control²⁸), *Helobdella triserialis* (negative control²³) and *Amphipholis squamata* (test case). Both the *Drosophila* and *Amphipholis* Engrailed fragments bound antibody on dot blots and competed with *Amphipholis* samples for antibody binding, whereas the *Helobdella* fragment did neither; both results suggest that the antibody 4D9 recognizes Engrailed protein in *Amphipholis*. Unfortunately, this antibody could not be used to examine *engrailed* expression in sea urchins, where an amino-acid substitution in the epitope interferes with binding²⁸. A polyclonal antibody raised to a fusion protein containing Orthodenticle¹⁹ from the sea urchin *Strongylocentrotus purpuratus* was used to analyse *orthodenticle* expression in a variety of echinoderms. A polyclonal antibody raised to a fusion protein containing Distal-less from the butterfly *Precis coenia* and having very broad phylogenetic cross-reactivity⁹ was used to analyse *distal-less* expression in echinoderms.

Immunolocalization. Samples were fixed in 4% formaldehyde in phosphate-buffered saline for 10–30 min at room temperature, washed, dehydrated and stored in 75% ethanol at –20°C. Blanking, probing and detection (diaminobenzidine/Ni²⁺) were performed as described²⁹. Samples were mounted in phosphate-buffered saline or cleared using a mixture of benzylbenzoate and benzyl alcohol before photography.

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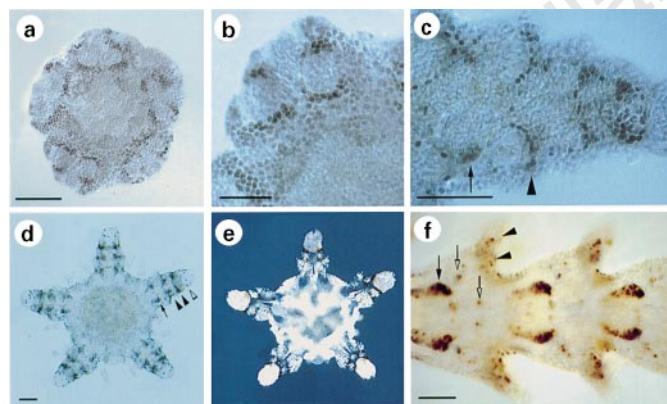


Figure 5 Expression of Engrailed protein in juvenile brittle stars (*Amphipholis squamata*). **a, b**, Pentagon stage, when skeletal ossicles are just beginning to form. Areas of active skeletogenesis, including the paired plates forming near future arms, are bounded by nuclei expressing *engrailed* protein. **c**, Arm tip of older juvenile, showing thin stripes of ectodermal expression (typically 1–2 cells wide; arrowhead) between forming ossicles. Stripes are transient, and fade within 3 arm segments proximally. Neuronal expression is out of the plane of focus (arrow). **d, e**, Juvenile under bright-field and polarizing light. Ectodermal stripes of expression are present between ossicles (filled arrowheads). A few nuclei have begun to express Engrailed protein where the boundary of the next arm segment will appear (open arrowhead). Neuronal expression is present in the older, more proximal arm segments (arrow). **f**, Decalcified arm of juvenile, showing neuronal expression primarily within repeated, paired ganglia (filled arrow), and in a few additional cells of unknown identity (open arrows). A few lateral cells within the ectoderm also express Engrailed protein, in areas between and adjacent to the lateral spine ossicles (arrowheads) as they form. Scale bars: **a, d, e**, 100 μ m; **b**, 50 μ m; **c, f**, 500 μ m.

Serrate2 is disrupted in the mouse limb-development mutant syndactylism

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The mouse *syndactylism* (*sm*) mutation impairs some of the earliest aspects of limb development and leads to subsequent abnormalities in digit formation^{1–3}. In *sm* homozygotes, the apical ectodermal ridge (AER) is hyperplastic by embryonic day 10.5, leading to abnormal dorsoventral thickening of the limb bud, subsequent merging of the skeletal condensations that give rise to cartilage and bone in the digits, and eventual fusion of digits. The AER hyperplasia and its effect on early digital patterning distinguish *sm* from many other syndactylies that result from later failure of cell death in the interdigital areas^{4,5}. Here we use positional cloning to show that the gene mutated in *sm* mice encodes the putative Notch ligand *Serrate2*. The results provide direct evidence that a Notch signalling pathway is involved in the earliest stages of limb-bud patterning and support the idea that an ancient genetic mechanism underlies both AER formation in vertebrates and wing-margin formation in flies^{6,7}. In addition to cloning the *sm* gene, we have mapped three modifiers of *sm*, for which we suggest possible candidate genes.

The adult phenotype of the *syndactylism* (*sm*) mutation, in its severest form, involves soft-tissue fusions along the entire length of the three middle digits, with distal skeletal elements converging into osseous fusions¹ (Fig. 1a). The earliest embryonic *sm* phenotype is a hyperplastic and irregular AER that is buried in the mesenchyme, rather than protruding above. It can be readily detected by whole-mount *in situ* hybridization using *Fgf8* as a marker (Fig. 1b, c) or by histological sections¹ (Fig. 1d, e).

As the first step towards positional cloning, we genetically mapped *sm* to distal chromosome 12 between the genetic markers D12Mit133 and D12Mit20 (Fig. 2) in an F₂ intercross (*sm* × *MOLF/Ei*) with 2,883 progeny, providing 5,766 informative meioses. We then constructed a yeast artificial chromosome (YAC) map of about 1.5 megabases (Mb) spanning the region. We generated 208 sequence-tagged sites (STSs), a subset of which we developed into genetic markers that recombined on either side of the mutation. This further narrowed the interval containing *sm* to about 300 kilobases (kb) within a single YAC of 365 kb (Y4; Fig. 2). Three STSs within the critical region showed DNA sequence similarity to members of the *Serrate* family of genes, which encode transmembrane-bound ligands of Notch characterized by epidermal growth factor (EGF) repeats⁸. Because the gene was distinct from the one previously identified vertebrate homologue of *Serrate*⁹, we refer to it as *Serrate2*. (Since our identification, the orthologous gene has been reported in rat¹⁰ and chick^{6,7}.)

Because Notch signalling pathways have been implicated in many cell-fate decisions and patterning processes, we tested whether *Serrate2* was a plausible candidate for the *sm* gene by virtue of its embryonic expression pattern. Whole-mount *in situ* hybridizations detect the *Serrate2* transcript throughout the limb ectoderm, but most strongly in the AER (Fig. 3a); similar results have been reported¹⁰ for the rat orthologue of *Serrate2*. Expression commences

with the formation of the early limb bud, and remains until the AER regresses. The gene is also expressed in somites, branchial arches, and tail ectoderm (Fig. 3a, b, and data not shown). Northern blots of adult brain RNA show two transcripts of 5.5 and 7.5 kb, neither of which displays a detectable difference between *sm* and wild type (data not shown).

We sequenced the 26-kb genomic region containing the wild-type *Serrate2* gene, as well as cDNAs covering most of the 3,744 bases of coding sequence of the wild-type transcript. Conceptual translation predicts a protein of 1,247 amino acids with a domain structure identical to the other members of the vertebrate family of *Serrate* genes^{9,10} (Fig. 3c). Certain aspects of the gene structure are noteworthy. Vertebrate *Serrate* genes have been described as containing 16 EGF repeats^{9,10}. Our genomic sequence reveals that nearly all of the 16 EGF repeats are encoded by single exons with introns occurring at conserved positions. Moreover, a partial EGF repeat comprising the carboxy-terminal portion of the *Delta/Serrate/Lag-2* homology domain¹¹ and a 17th highly divergent full-length EGF repeat are suggested by conserved intron positions within the amino-acid alignment (Fig. 3c).

To search for a coding mutation in *Serrate2*, we sequenced all

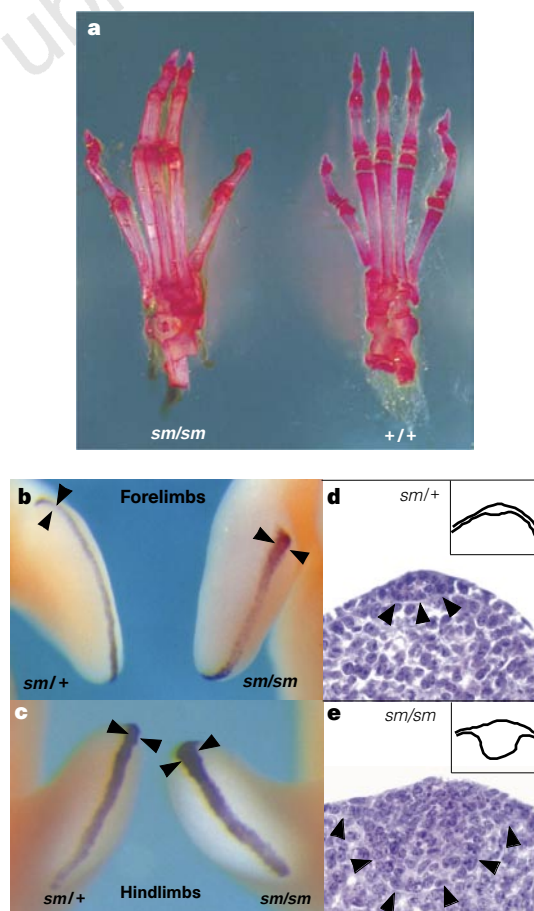


Figure 1 a Skeletal preparations of an *sm*-homozygous (*sm/sm*) hind foot and a wild-type (+/+) hind foot stained by Alizarin red. Note the osseous fusions of phalanges in digits 3 and 4 of the *sm* homozygote; when such fusions occur, digits 2–4 are always tightly joined by soft tissue, which was digested away in this preparation. b–e, Hyperplasia of the *sm* AER shown by comparison of *sm/+* and *sm/sm* E11.0 limbs. *Fgf8* expression in forelimb buds (b) and hindlimb buds (c) show the widened AER at the surface (arrowheads). Haematoxylin and eosin-stained histological cross sections show the AER (arrowheads), which is greatly enlarged and protrudes further into the mesenchyme in *sm/sm* mice (e) than in *sm/+* mice (d).

coding exons and intron–exon boundaries of the gene in *sm* mice and the parental strain on which *sm* arose. We found a single G-to-A missense mutation (Fig. 3d) in *sm* mice that changes an invariant glycine to serine at codon 267 in the first EGF repeat (Fig. 3e). We conclude that *Serrate2* is the *sm* gene, because it maps to the critical interval; it shows expression in the developing limb consistent with the *sm* phenotype; and, most importantly, it has a missense mutation present in *sm* but absent in the parental strain.

Evolutionary, structural and genetic evidence show that the altered glycine residue in the EGF repeat has functional importance. First, the glycine in this position has been conserved over a total of three billion years of divergence among all known orthologous EGF repeats (Fig. 3e), with the only other invariant residues being the seven cysteines and the two other glycines. Second, the solution structure of a more divergent EGF repeat (Prosite accession no. PDOC00021) shows that the glycines are situated in tight turns, allowing disulphide bonding between the flanking cysteines¹², and that the addition of a side chain—even a small one, such as serine—may interfere with proper folding. Third, five known missense mutations alter glycine residues in EGF repeats of *Notch* and *Delta* in *Drosophila* and *lag-2* in *Caenorhabditis elegans*^{11,13–15}. Two of these, *Df^{sup4}* and *Ax¹⁶¹⁷²*, affect the homologous glycine; both are hypomorphic rather than null mutations, suggesting that *sm* may also be a hypomorph.

In our (*sm* × *MOLF/Ei*) mapping cross, we noticed that the *sm* phenotype was strongly modified by genetic background. The inbred *sm* strain shows a uniform phenotype with the middle three digits tightly fused by soft tissue syndactyly but without osseous fusions, whereas the *sm* homozygotes from the F₂ intercross displayed much greater phenotypic variation. Of 519 *sm* homozygotes, 19% were unaffected, 65% displayed a weak phenotype (noticeably less extreme than in the *sm* inbred strain, ranging from barely detectable soft-tissue webbing to incomplete fusions of three toes on each foot), and 16% displayed a severe phenotype (at least as extreme as in the *sm* inbred strain, completely affecting all three middle toes of each foot and often involving osseous fusions). The strong influence of genetic background further suggests that *sm* may be a hypomorphic mutation, for which the phenotype depends sensitively on levels or allelic variations in interacting proteins.

To search for modifier loci that might account for the phenotypic variability, we performed a genome-wide linkage analysis (Fig. 4). It revealed striking evidence for a major modifier on chromosome 11, with the *MOLF/Ei* allele acting as a suppressor (lod score = 7.8.

genome-wide *P*-value < 10^{−4}). An enhancer locus on chromosome 4 and a suppressor locus on chromosome 16 also showed statistically significant evidence of linkage, just above the genome-wide threshold for significance (lod score = 4.1, genome-wide *P*-value = 0.05). There was also suggestive evidence for modifiers on chromosomes 6 and 7, with respective lod scores of 3.9 and 3.5. The cross thus revealed at least three modifier loci, which, in analogy to the classical modifier genetics in the *Drosophila* Notch pathway, may be genes involved in the *Serrate2* pathway or other aspects of vertebrate limb development.

Examination of the mouse gene map¹⁶ suggests some interesting candidates. Most intriguingly, the enhancer on chromosome 4 maps to the region containing the *Polysyndactyly* (*Ps*) mutation, which causes hypertrophy of the AER by downregulating cell death¹⁷. A weak allele at this locus might well exacerbate the AER hypertrophy caused by *sm*. The suppressor on chromosome 11 maps to a region containing several transcriptional regulators (*Lim1*, *Dsh2*, *Evi2*, *Mox1* and the *HoxB* cluster), although none have yet been implicated in limb patterning or a Notch signalling pathway. The suppressor on chromosome 16 is closely linked to *Hes1*, which is a member of a large family of genes homologous to *Drosophila Enhancer of split*. Transcription of *Hes1* has been shown to be activated by Notch1, the likely receptor for *Serrate2* in the AER^{10,18}. Further candidates may become apparent as increasing numbers of mouse and human cDNAs are mapped to specific chromosomal locations. Definitive identification of the modifier loci will require careful characterization of sequence and expression variation and, eventually, transgenesis experiments.

Serrate2 is expressed in many tissues including somites, branchial arches, tail ectoderm, surface ectoderm, thymus, salivary glands, cranial ganglia, dorsal root ganglia, and adult brain (ref. 10 and our data). We therefore performed an extensive histological analysis of *sm* embryos at embryonic day 11 (E11) and adults to look for defects other than in the developing limb, but none was evident. As shown in the original description of *sm*, the only other visible phenotype is tail kinking¹, the incidence of which fell from 40% to 20% upon outcrossing *sm*. In the current *sm* inbred strain, which was derived from further outcrosses, it is only 2–5%. It was also noted¹ that a subset of animals showed hyperplasia of tail and non-AER limb ectoderm, but we found no evidence of this in the current *sm* strain. The absence of other detectable defects could be due to the *sm* mutation being a hypomorph (with limb development being most sensitive to the partial loss of function) or to *Serrate2* being fully dispensable in other tissues (perhaps owing to redundancy with *Serrate1*, with which it is coexpressed in some tissues¹⁰). Additional defects might conceivably be seen on other genetic backgrounds, as suggested by the existence of modifiers of both the limb and tail-kink phenotypes.

Understanding of the developmental function of *Serrate* genes has come primarily from studies of the *Drosophila* wing margin. Like the vertebrate AER, this specialized structure forms at the dorsoventral boundary of the incipient appendage and regulates its outgrowth and patterning. It is formed by cells expressing the Notch receptor, the response of which to its ligands, *Serrate* and *Delta*, is modulated by the *fringe* gene product¹⁹. The finding that a mutation in *Serrate2* affects the AER in the mouse, together with recent reports^{6,7} that ectopic expression of a *fringe* homologue can cause ectopic or otherwise aberrant formation of the AER in the chick indicate that a Notch pathway and *fringe* genes are also involved in the formation of the vertebrate AER. Overlapping expression patterns of vertebrate homologues in the branchial arches (*Radical fringe* and *Serrate2*), and in the hindbrain (*Lunatic fringe* and *Serrate1*)^{6,7}, provide further circumstantial evidence for a conserved interaction between *fringe* homologues and Notch pathways. The *fringe/Notch* interaction, as well as pathways involving homologues of *hedgehog*, *decapentaplegic* and *wingless*, may thus be examples of ancient developmental mechanisms that have been co-opted several

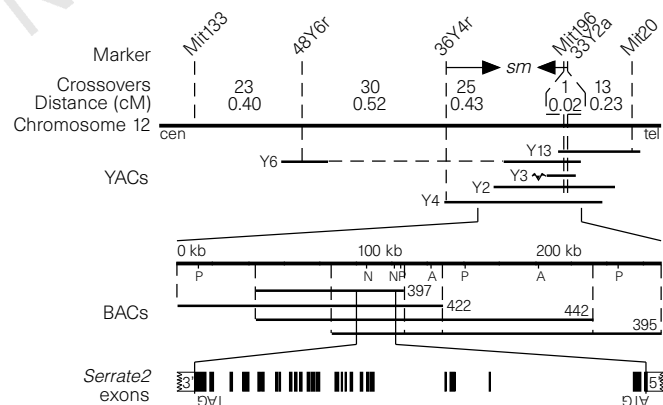


Figure 2 Genetic and physical maps of the *Serrate2* region. Markers 44Y6r, 36Y4r and 33Y2a were developed by subcloning the YACs shown. We used 49 YAC-derived STSs to generate this map. The broken line indicates deletion in Y6, the jagged line indicates chimaerism in Y3. Genetic and YAC maps are proportional to genetic distances; BAC map and *Serrate2* intron–exon structure are proportional to physical distances as determined by restriction mapping (BAC) or sequence (*Serrate2*). Restriction sites: A, *AscI*; N, *NotI*; P, *PacI*.

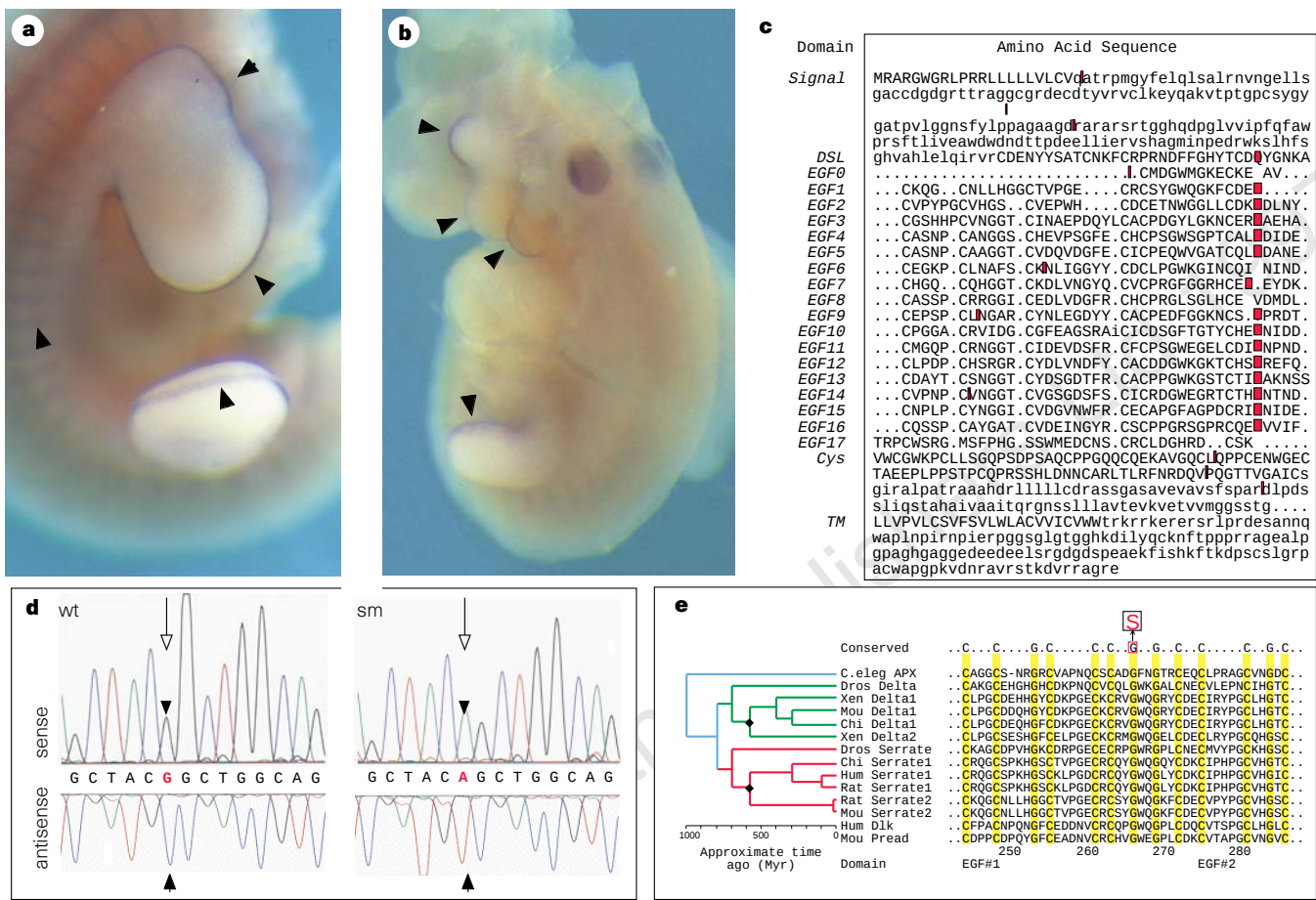


Figure 3 **a**, Expression of *Serrate2* in the AER, other areas of the limb ectoderm, and somites (arrowheads) in the E11.0 embryo. **b**, Expression of *Serrate2* in the forelimb and branchial arches (arrowheads) of the E10.0 embryo. **c**, deduced amino-acid sequence of mouse *Serrate2* with positions of introns shown as red blocks. Note the conserved positions of introns in the EGF repeats, which are numbered on the left. The sequence is contiguous with the exception of the insert in the 10th EGF repeat (denoted by 'i'). Uppercase sequences are, in order, inferred signal peptide (signal), *Delta/Serrate/Lag-2* homology domain (DSL), EGF repeats, cysteine-rich region (Cys), and transmembrane domain (Tm). **d**, Sequence chromatograms showing the wild-type sequence (left) and the *sm* sequence (right). The affected base is indicated by arrows in forward (top) and reverse (bottom) directions. The mutation changes codon 267 from glycine (GGC)

to serine (AGC). **e**, Alignment of the first EGF repeats of all known DSL proteins (except Lag-2, which does not contain this repeat), indicating conserved residues and the *sm* mutation. Numbering is according to the mouse *Serrate2* amino-acid sequence. The evolutionary tree on the left depicts the sequences' relationships, as determined by analysis of the DSL domain and the first two EGF repeats, which are the unambiguously homologous domains of these proteins. Green, *Delta* subtree; red, *Serrate* subtree. Carets denote gene duplication events. Human Dlk and mouse preadipocyte factor do not contain a DSL domain (and are therefore not included in the tree) but do have this EGF repeat. The tree is unrooted as *Delta* or *Serrate* could be orthologous or paralogous to *C. elegans* APX. Approximate time scale is according to an earlier study²³.

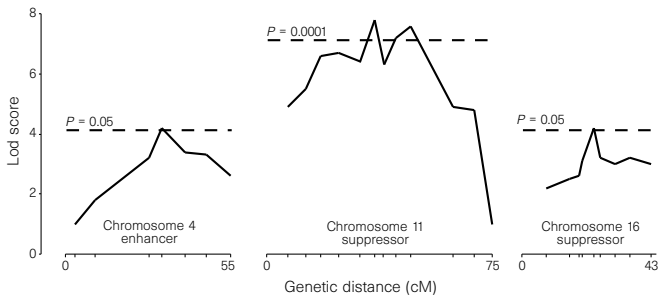


Figure 4 Results from genome scan for suppressors and enhancers in the *sm* mapping cross. Tickmarks indicate positions of markers typed. Vertical scale denotes lod score, broken lines indicate *P*-values for assessing the genome-wide statistical significance of the highest lod score.

times in metazoan evolution to cooperate in the formation of independently evolved morphological structures, such as the fly wing and vertebrate limb. □

Methods

Genetic and physical mapping. *Syndactylism* arose spontaneously 46 years ago in the A inbred strain¹. It was subsequently outcrossed and inbred to form its own stock. Males homozygous for *sm* were mated to *MOLF/Ei* females (Jackson labs), and *F*₁ progeny were crossed *inter se*. Critical recombinants were backcrossed to *sm* homozygotes to infer the *sm* genotype. Polymerase chain reaction (PCR) genotyping was done by standard protocols²⁰, with flanking markers D12Mit133 and D12Mit20 and recombinationally inseparable marker D12Mit196. We screened a YAC library with all three markers, but, as 5,766 meioses should give a very small critical interval, we focused on YACs containing D12Mit196. We isolated five such YACs on a pulsed-field gel, partly *Sau3AI*-digested them, generated 208 STSs by subcloning and sequencing, and developed 20 of these into genetic markers polymorphic between *MOLF/Ei* and *sm*. One YAC, 405D12, of 365 kb, contained genetic markers

flanking *sm* and thus the entire critical interval. Polymorphic markers were used to screen a bacterial artificial chromosome (BAC) library to isolate clones partly spanning the *sm* region.

Genomic sequencing. An M13 library from BAC 442 was prepared and partly sequenced to yield 26 kb of genomic sequence spanning the coding region of *Serrate2*. We designed ten sets of nested PCR primers covering the coding region and amplified corresponding fragments from *sm* and its parental strain; the fragments were sequenced using internal primers.

cDNA sequencing. The wild-type cDNA sequence covering nucleotides 185–3,475 was determined independently by sequencing a cDNA clone from an E11.5 whole-embryo library cloned in bacteriophage lambda (Novagen), as well as full-insert sequencing of two clones containing ESTs from *Serrate2* from the IMAGE collection (Genbank accession nos W13776 and AA024229). The mutation found in genomic DNA was confirmed by sequencing cDNA from *sm* mice. Reverse transcription with gene-specific primers was performed with Superscript (BRL) on 100 ng of poly(A)⁺ RNA selected with oligo dT columns (Stratagene) from total RNA isolated by Trizol (BRL) homogenization of brains of A and *sm* females.

Northern analysis. Poly(A)⁺ RNA (10 µg) from *sm* and Balb/C female brains, isolated as described above, was separated on a Glyoxal gel, blotted and probed with the lambda clone by standard procedures²¹.

Whole-mount in situ hybridizations. Embryos were from timed Balb/C (wild type) or *sm*/+ × *sm*/*sm* matings; embryos from the latter were genotyped using the recombinationally inseparable marker D12Mit196 to infer *sm* genotype. Digoxigenin-labelled (Boehringer) probe was generated with T3 RNA polymerase (Ambion) from EST clone W13776, or with T7 RNA polymerase (Ambion) from the lambda clone, to which a T7 promoter was added by PCR. *In situ* hybridizations were performed by standard protocol²² at high stringency without an RNase step.

Genome scan for modifier loci. PCR genotyping was performed as above. Several hundred genetic markers were tested for polymorphism between *MOLF/Ei* and *sm*; 235 were found to yield differences detectable on MetaPhor (FMC) agarose gels. First, the markers were genotyped on five DNA samples; pooled DNA from severely affected *sm* homozygotes from the F₂ cross; pooled DNA from unaffected *sm* homozygotes from the same cross; an F₁ heterozygote; and the two parental strains. Seven regions potentially containing candidate genes were identified by skewed signal intensity of the two alleles in one or both pools, as compared to the F₁ control. The regions were followed up by individually genotyping all 96 unaffected and all 84 severely affected *sm* homozygotes. Five regions showed allelic skewing indicating the presence of a modifier locus; three surpassed the threshold for genome-wide significance (see below). To exclude that allelic skewing resulted from segregation distortion, we genotyped 137 randomly chosen *sm*/+ and +/+ progeny with markers in these three regions; no skewing was observed. Markers for the three regions were: D4Mit235, 192, 288, 81, 153, 175, 279; D11Mit80, 205, 270, 23, 242, 298, 245, 178, 285, 330, 333, 302; D16Mit183, 28, 209, 101, 57, 58, 84, 47, 203.

Linkage analysis. For each marker, we compared the hypothesis that extreme phenotypes (unaffected versus severely affected) depend on marker genotype, according to penetrance parameters p_{AA} , p_{AB} , p_{BB} , with the null hypothesis that phenotype is independent of marker genotype, according to a single penetrance parameter p . For each marker, we calculated the maximum-likelihood estimates of these parameters and the corresponding lod score for comparing the hypotheses. For a search across all autosomes (excluding chromosome 12, on which *sm* resides), the threshold for genome-wide significance at the $P = 0.05$ level is a lod score of 4.1. Suppressor loci were defined as those showing an excess of *MOLF/Ei* alleles in unaffected mice and a deficit in severely affected mice. Enhancer loci were those with the opposite effect.

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Severe neuropathies in mice with targeted mutations in the ErbB3 receptor

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Neuregulins and their specific receptors, members of the ErbB family of tyrosine kinases, have been implicated in the control of growth and development of Schwann cells^{1–3}, specialized cells that wrap around nerve axons to provide electrical insulation. Here we use gene targeting to generate mice that lack ErbB3, a high-affinity neuregulin receptor^{4–6}. Homozygous *erbB3* mutant embryos lack Schwann-cell precursors and Schwann cells that accompany peripheral axons of sensory and motor neurons. The initial development of motor neurons and sensory neurons of dorsal root ganglia occurs as it should, but at later stages most motor neurons (79%) and sensory neurons in dorsal root ganglia (82%) undergo cell death in *erbB3* mutant embryos. Degeneration of the peripheral nervous system in *erbB3* mutant pups is thus much more severe than the cell death in mice that lack neurotrophins or neurotrophin receptors^{7,8}. We also show that ErbB3 functions in a cell-autonomous way during the development of Schwann cells, but not in the survival of sensory or motor neurons. Our results indicate that sensory and motor neurons require factors for their survival that are provided by developing Schwann cells.

The *erbB3* gene encodes a member of the ErbB family of tyrosine-kinase receptors that has unusual biochemical features. Four amino

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acids that are either highly conserved or invariant in the catalytic domains of protein kinases are altered in ErbB3, and the protein has little or no tyrosine kinase activity. ErbB3 binds neuregulins with high affinity but requires co-receptors like ErbB2 to transmit the neuregulin signal^{15,6}. Mouse strains carrying two distinct mutant *erbB3* alleles were generated by gene targeting in embryonic stem (ES) cells. In the *erbB3*^Δ allele, exons encoding a large part of the extracellular domain of ErbB3 were deleted and replaced by a neomycin-resistance (*neo*) cassette (Fig. 1a, c). Homozygous mutant mice produce a truncated ErbB3 protein of relative molecular mass 120K, detectable by western blot analysis (Fig. 1d), which was not phosphorylated on tyrosine residues (data not shown). In a second allele, the *neo* gene was directly fused to an exon encoding extracellular sequences of ErbB3, thereby introducing termination codons in all three reading frames (Fig. 1b, c). No ErbB3 protein is produced from this allele, as shown by western blotting (Fig. 1d), and we named that allele *erbB3* null (*erbB3*⁻). Animals carrying homozygous *erbB3*^Δ or *erbB3*⁻ alleles had identical phenotypes.

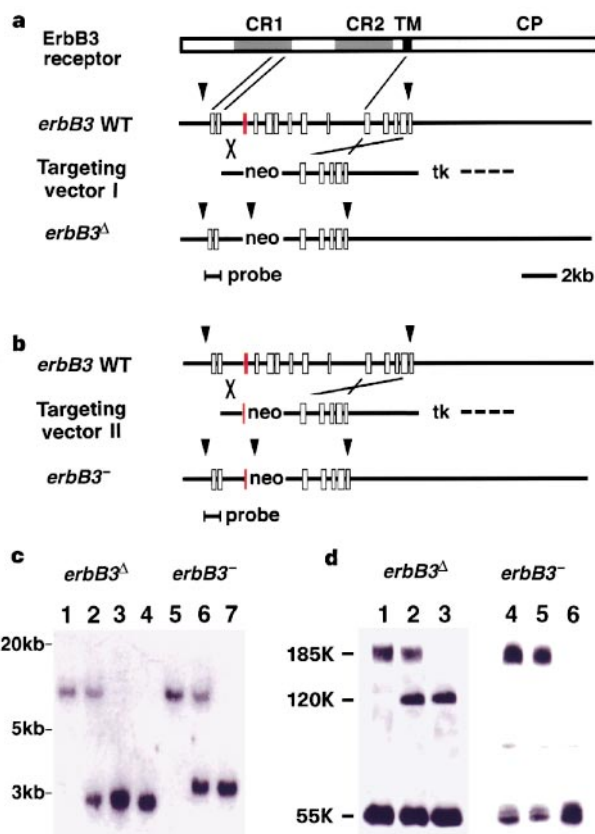


Figure 1 Strategy applied to mutate the mouse *erbB3* gene and effect of the mutations. **a**, Top, The ErbB3 receptor (CRI and CRII, cysteine-rich segments I and II; TM, transmembrane, CP, cytoplasmic domains). The wild-type *erbB3* gene, the *erbB3*^Δ targeting vector (deleted sequences correspond to nucleotides 1,308–2,112 in the cDNA²⁶), and the mutant *erbB3*^Δ alleles are shown below; arrowheads indicate *Eco*RI restriction sites. **b**, The wild-type *erbB3* gene, the *erbB3*⁻ targeting vector (deleted sequences correspond to nucleotides 1,343–2,112 in the cDNA²⁶) and the mutant *erbB3*⁻ allele. The red exon encodes amino acids 352–375 and is deleted in the *erbB3*^Δ allele. In the *erbB3*⁻ allele, this exon is fused directly to the *neo* gene. **c**, Southern blot analysis of genomic DNA digested with *Eco*RI from: lane 1, wild type; lane 2, *erbB3*^{Δ/+}; lane 3, *erbB3*^{Δ/erbB3} embryos; lane 4, *erbB3*^{Δ/erbB3} ES cells; lane 5, wild type; lane 6, *erbB3*^{-/+}; lane 7, *erbB3*^{-/-} embryos. **d**, Western blot analysis of ErbB3 done on anti-ErbB3 immunoprecipitates of total protein extracts from E12.5 embryos: lane 1, wild type; lane 2, *erbB3*^{Δ/+}; lane 3, *erbB3*^{Δ/erbB3}; lane 4, wild type; lane 5, *erbB3*^{-/+}; lane 6, *erbB3*^{-/-}. Wild-type ErbB3 (185K); *erbB3*^Δ (120K); IgG heavy chain (55K).

Mice heterozygous for *erbB3*^Δ or *erbB3*⁻ were healthy and fertile. Matings between heterozygous animals produced homozygous newborn pups at a reduced frequency (21% of the number expected; Table 1). Dissection of embryos at different stages of development indicated that most homozygous embryos died between E11.5 and E13.5 (Table 1); surviving embryos developed to term. Compared to control littermates, newborn homozygous pups were normal in gross anatomy, slightly reduced in size (averaging 10% weight

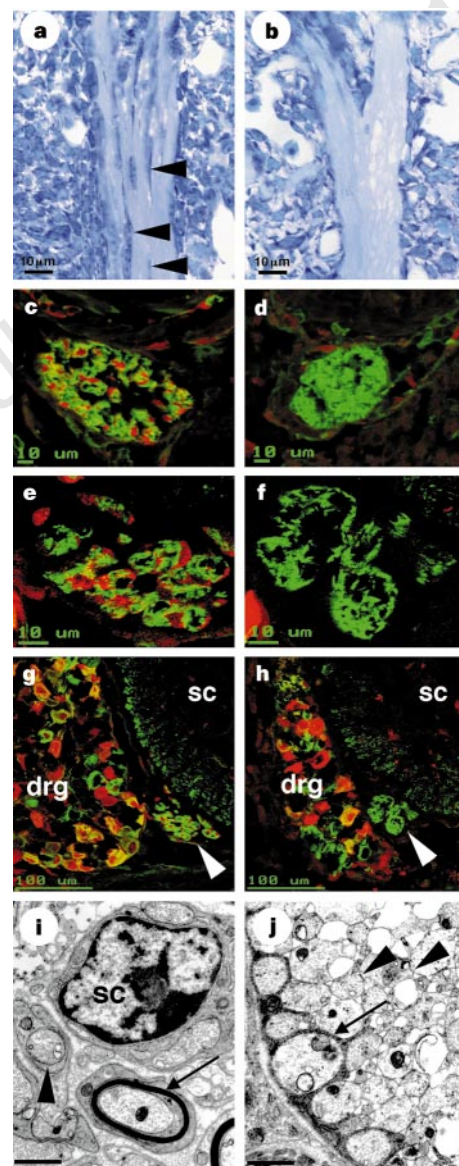


Figure 2 ErbB3 mutant embryos lack Schwann cells and their precursors. **a**, **b**, Histological appearance of a spinal nerve from E12.5 *erbB3*^{Δ/+} (**a**) and *erbB3*^{Δ/erbB3} (**b**) embryo (toluidine-blue staining). Arrows point towards Schwann-cell precursors. **c–h**, Double-immunofluorescence labelling with anti-S100 antibody (red) and anti-neurofilament protein (NF160) antibody (green) of E16.5 *erbB3*^{Δ/+} (**c**, **e**, **g**) and *erbB3*^{Δ/erbB3} (**d**, **f**, **h**) embryos. **c**, **d**, N. vagus; **e**, **f**, axons of ventral root motor neurons. Overview (**g**, **h**) showing dorsal root ganglia (drg), spinal cord (sc) and ventral roots (arrowheads). **i**, **j**, Electron micrographs of ventral roots of *erbB3*^{Δ/+} (**i**) and *erbB3*^{Δ/erbB3} (**j**) embryos (E18.5); in (**i**), Schwann cells (sc) that wrap axons (arrowheads) or begin to form myelin sheets (arrow) are indicated; in **j**, axonal membranes in direct contact (arrowheads) and fibroblasts of the epineurium, which wrap axons at the edge of the nerve (arrow). Scale bars in **i**, **j**: 1 μm.

reduction) and cyanotic, although their heart continued to beat for some time after birth. The homozygous mutant pups did not move or react to tactile stimulation; they did not breathe and lung alveoli did not expand. Mutant E16.5 embryos, however, did respond to tactile stimulation, although spontaneous movement was reduced.

Histological analysis of homozygous *erbB3^Δ/erbB3^Δ* or *erbB3^{-/-}*

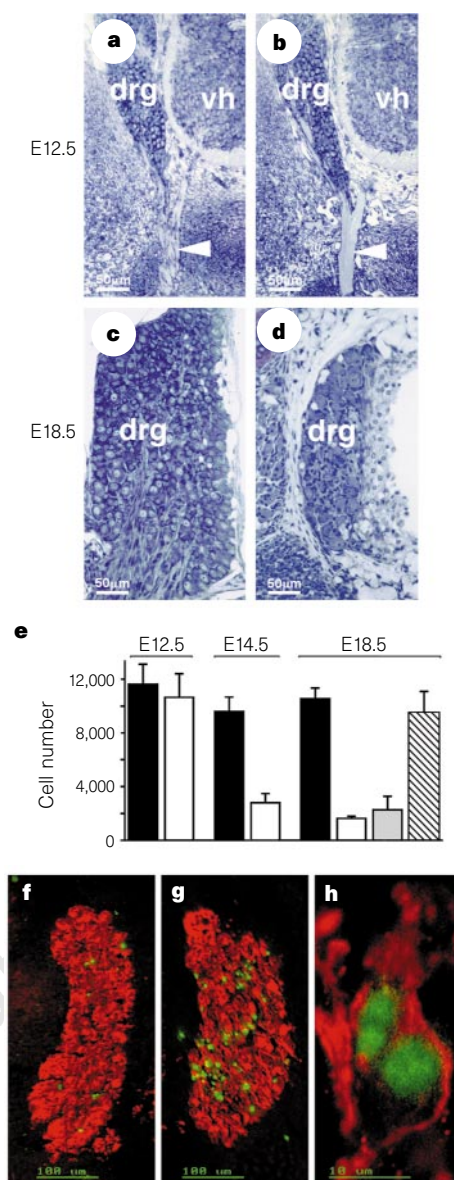


Figure 3 Loss of sensory neurons in *erbB3* mutant embryos. **a–d**, Histology of control (**a, c**) and *erbB3^Δ/erbB3^Δ* (**b, d**) embryos on E12.5 (**a, b**) and E18.5 (**c, d**) (toluidine-blue staining). Ventral horn (vh); dorsal root ganglia (drg); arrowheads point towards spinal nerves. **e**, Sum of cell numbers in L4 and L5 dorsal root ganglia on E12.5 (black, control; white, *erbB3^Δ/erbB3^Δ*) and sum of neuron numbers in L4 and L5 dorsal root ganglia on E14.5 or E18.5 (black, control; white, *erbB3^Δ/erbB3^Δ*; grey, *erbB3^{-/-}*; cross-hatched, chimaeras containing wild-type and *erbB3^Δ/erbB3^Δ* cells). Neurons were identified using the histological criteria described in Methods. Mean numbers $\pm$ s.d. are shown (n is 3–5 embryos in each group). Cell-diameter measurements indicated that the smallest and largest neurons were preferentially lost; muscle spindles were also absent, indicating a total loss of proprioceptive neurons. **f–h**, TUNEL (TdT-mediated dUTP nick end labelling) staining of control (**f**) and *erbB3^{-/-}* (**g, h**) dorsal root ganglia on E13.5 using a FITC-labelled anti-digoxigenin antibody (green); sections were counterstained with anti-peripherin (red); **h**, an apoptotic cell at higher magnification.

embryos on E12.5 demonstrated the presence of peripheral nerves but the absence of Schwann-cell precursors accompanying these nerves (Fig. 2b), which can be seen in the control embryo (arrowheads in Fig. 2a). A lack of Schwann-cell precursors accompanying the vagus nerve or motor neurons was also evident from immunohistological analysis with S-100-specific antibodies (E16.5), a widely used marker for Schwann cells² (compare Fig. 2f, d with c, e). Likewise, *in situ* hybridization with p75-, P0- (E12.5) or Krox-20-specific probes (E16.5) revealed that there were no Schwann-cell precursors accompanying sensory or motor neurons (not shown). S-100 staining showed that glial cells in the enteric nervous system were also absent (not shown), whereas other types of glial cells, such as supporting cells in dorsal root ganglia or glia in the central nervous system, appeared normal (Fig. 2g, h). Electron micrographs of the ventral roots in the subarachnoid space of E18.5 embryos revealed closely packed axonal membranes not separated by Schwann cells (arrowhead, Fig. 2j), but the epineurium was present (arrow, Fig. 2j). In the control, axons are wrapped by myelinating (arrow, Fig. 2i) and non-myelinating Schwann cells (arrowhead, Fig. 2i). *ErbB3* is therefore essential for development of Schwann cells that accompany peripheral nerves; Schwann cells strongly express this receptor^{9,10}. *ErbB3* mediates a signal given by sensory and motor neurons, which express the specific ligand neuregulin-1 (refs 1, 10–12).

In the sensory nervous system of *erbB3^Δ/erbB3^Δ* or *erbB3^{-/-}* embryos, dorsal root ganglia form but subsequently degenerate. Thus, on E12.5 the dorsal root ganglia of mutant embryos are normal in their histological appearance (Fig. 3a, b) but are much smaller at later stages (Fig. 3c, d). The number of cells in dorsal root ganglia was initially similar in control and mutant embryos on E12.5; on E14.5 and on E18.5, neurons were reduced in number by 70 and 82%, respectively (Fig. 3e). TUNEL staining indicated that apoptosis increased in dorsal root ganglia of mutant E13.5 embryos; most apoptotic cells (83%) were positive for peripherin and so correspond to postmitotic neurons¹³ (Fig. 3f–h). We conclude that the majority of cells undergo cell death in dorsal root ganglia of *erbB3* mutant embryos after these ganglia have formed and that most of the dying cells are sensory neurons. In contrast, cranial ganglia and cranial nerves in mutant embryos were abnormal by E10 when these structures form (results not shown). This early defect is also present in neuregulin-1-deficient and *erbB2^{-/-}* embryos and was attributed to the absence of neurons deriving from hindbrain neural crest but not to cell death of postmitotic sensory neurons^{9,10,14}.

Motor neurons in *erbB3^Δ/erbB3^Δ* or *erbB3^{-/-}* embryos also start to form but subsequently degenerate. Thus on E12.5 or E16.5, motor neurons in the ventral horn (vh) of the spinal cord appear histologically normal (Fig. 3a, b); we also observed normal responses to muscle innervation, like the clustering of acetylcholine receptor on E15.5 (by staining with α -bungarotoxin; Fig. 4a, b) or the induction of the α -subunit of acetylcholine receptor in postsynaptic nuclei on E15.5 (by *in situ* hybridization; results not shown). In contrast, on E18.5 the numbers of motor neurons in the ventral horn of the spinal cord were markedly reduced and the diameter of ventral roots leaving the spinal cord was much smaller in the mutant embryos (arrowheads, Fig. 4c, d). From electron micrographs of ventral roots, a loss of 79% of motor axons was recorded in mutant embryos (Fig. 4e).

To determine whether *ErbB3* functions directly in transducing trophic signals in neurons, we generated chimaeric animals by injection of *erbB3^Δ/erbB3^Δ* embryonic stem cells into wild-type blastocysts and analysed to what extent wild-type and mutant cells colonized dorsal root ganglia and other tissues. ES and blastocyst-derived cells carry different alleles of glucose phosphate isomerase (GPI) which can be distinguished electrophoretically¹⁵. We found that GPI isoenzyme ratios in dorsal root ganglia and other organs (for instance liver, skin, spinal cord, dorsal root ganglia) from

chimaeric pups were similar (Fig. 5). In contrast, sciatic nerve extracts rich in Schwann-cell cytoplasm had significantly less GPI^a-isoenzyme derived from mutant cells. We isolated Schwann-cell precursors from explant cultures of dorsal root ganglia of chimaeric mice; extracts from these cells contained virtually no GPI^a (Fig. 5). The overall appearance of dorsal root ganglia and ventral roots was essentially normal even in chimaeras with very high (for example, 90%) ES-cell contributions (Fig. 3e, and data not shown). We conclude that ErbB3 functions cell-autonomously in the development of Schwann cells but not in the survival of sensory or motor neurons. This agrees with the *erbB3* expression pattern, as determined by *in situ* hybridization: *erbB3* is strongly expressed in developing Schwann cells but not in motor neurons and is found only in a subset of cells in dorsal root ganglia¹⁰.
Neuregulins and their receptors have also been implicated in epithelial growth and differentiation^{16–18}. We have not seen any obvious developmental defects in epithelia of *erbB3* mutant embryos. Nerve targets like skin or skeletal muscle also appeared histologically normal and skeletal muscle from mutant E18 embryos

contracted normally upon direct electrical stimulation *in vitro*; the gross anatomy of the brain was unaffected by the *erbB3* mutation. In contrast, mutant embryos displayed marked changes in the development of sympathetic ganglia that appeared early in development. The perinatal death and severe neurodegeneration of *erbB3* mutant animals precluded analysis of other possible roles for instance in the function of neuromuscular synapses^{19,20} or differentiation of the mammary gland¹⁸. When analysed by *in situ* hybridization, developing Schwann cells express ErbB3 but not ErbB4, and therefore only one of the high-affinity receptors for neuregulins. Skeletal muscle and epithelial cells express ErbB3 and ErbB4 which might take over similar functions in these cell types.
The phenotype of *erbB3* mutant mice highlights the importance of neuregulins and their receptors in the development of Schwann cells of the peripheral nervous system. In E10.5 embryos carrying mutant *erbB3*, *erbB2* or neuregulin genes, Schwann-cell precursors that associate with the developing spinal nerves and express genes typical for the neural crest are present, but are already severely reduced in number^{9,10}. Thus, changes in the development of

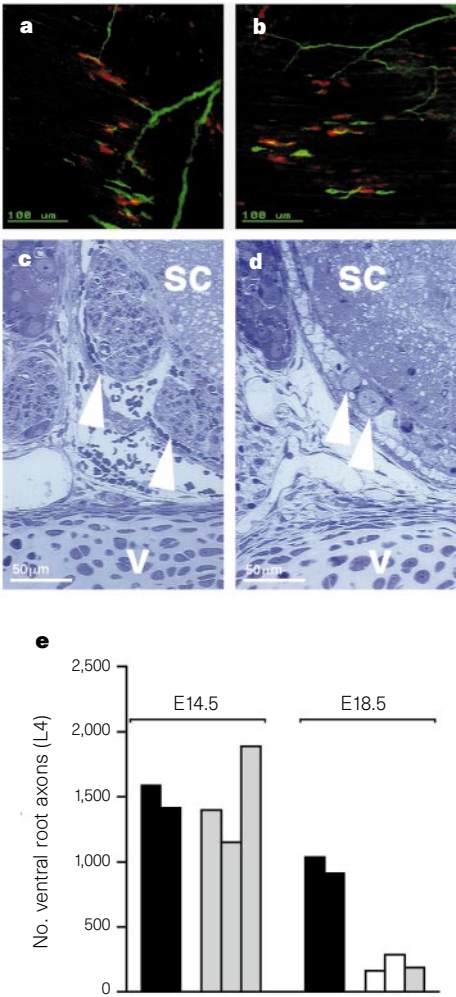


Figure 4 Loss of motor neurons in perinatal *erbB3* mutant embryos. **a, b**, Neuromuscular junctions in intercostal muscle of control (**a**) and *erbB3*^{-/-} (**b**) embryos on E15.5 were visualized by double immunofluorescence analysis using α -bungarotoxin (red) and anti-NF160 antibodies (green). **c, d**, Semithin sections of control (**c**) and *erbB3*^Δ/*erbB3*^Δ (**d**) embryos at E18.5 (toluidine-blue staining). Arrowheads point towards the ventral roots (L4). Spinal cord (sc) and vertebrae (v) are indicated. **e**, Numbers of motor axons present in the ventral roots of individual control and mutant embryos at indicated developmental stages (black, control; grey, *erbB3*^{-/-}; white, *erbB3*^Δ/*erbB3*^Δ).

Table 1 Viability of *erbB3* mutant embryos

Age	Genotype of live embryos			Genotype of live embryos		
	+/+	<i>erbB3</i> ^{Δ/+}	<i>erbB3</i> ^{Δ/Δ}	+/+	<i>erbB3</i> ^{-/+}	<i>erbB3</i> ^{-/-}
E11.5	9	17	8 (92%)	8	19	12 (133%)
E12.5	23	46	14 (61%)	17	33	14 (84%)
E13.5	27	52	7 (27%)	8	18	2 (23%)
E16.5	59	115	13 (22%)	38	76	10 (26%)
E18.5	40	81	9 (22%)	17	33	4 (24%)
Newborn pups	57	114	21 (21%)	43	87	9 (21%)

Analysis of *erbB3* genotypes of live embryos and newborn pups generated from intercrosses of heterozygous animals carrying *erbB3*^Δ or *erbB3*⁻ alleles. Numbers in parenthesis indicate the percentage of live homozygous mutant embryos compared to the expected mendelian ratio. Genotypes were identified by PCR analysis on DNA isolated from embryos between E11.5 and E18.5 or from newborn pups. In addition to live embryos, many embryos without heartbeat or with signs of resorption were seen, most of which had a homozygous mutant genotype.

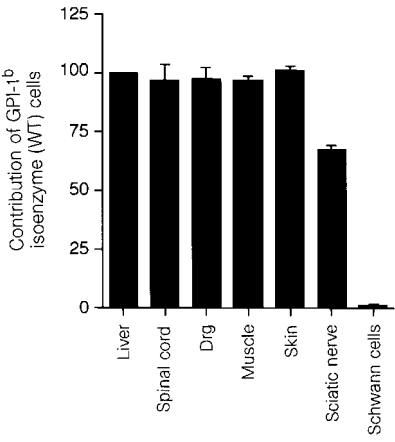


Figure 5 Contributions of wild-type and *erbB3*^Δ/*erbB3*^Δ cells to various tissues of chimaeric animals. Chimaeras were produced by injection of *erbB3*^Δ/*erbB3*^Δ ES cells (derived from 129 mice that express GPI^a) into wild-type blastocysts (derived from C57BL/6 mice that express GPI^b). Highly chimaeric animals were chosen to determine the isoenzyme ratios of GPI in various tissues of the chimaeras. The wild-type (GPI^b) contribution to the liver was normalized to 100%. Contributions of wild-type cells to other tissues are displayed as a percentage of the liver contribution; mean numbers $\pm$ s.d. are shown ($n = 2$ –3 embryos in each group).

Schwann-cell precursors are apparent before genes specific for Schwann cells (like S-100 or Krox-20) are expressed. Schwann cells *in vitro* or *in vivo* have been reported to produce a variety of neurotrophic factors like CNTF, GDNF, BDNF, LIF, PDGF, FGF, NT-3 or TGF- β and may support development, maintenance and regeneration of the peripheral nervous system (for a review, see ref. 21). Mutations in mice and other mammals that affect Schwann-cell function and differentiation have been described; also transgenes that interfere with Schwann-cell function and differentiation have been introduced into mice^{22–25}. Such animals have mild neuropathies. Our animal model lacks developing Schwann cells that associate with sensory and motor neurons. Most sensory and motor neurons degenerate in such animals mutant for *erbB3*. However, *ErbB3* does not directly transduce trophic signals in sensory and motor neurons, neuronal degeneration being caused indirectly: we suggest that it is the absence of Schwann cells that causes the neurodegeneration. Alternatively, neuregulins might regulate expression of neurotrophic factors in peripheral tissues that express *ErbB3*, like muscle or epidermis. Sensory and motor neurons of *erbB3* mutant animals die during distinct developmental periods: neuronal cell death peaks between E12 and E14 in sensory neurons, and between E15 and E18 in motor neurons. The extent and temporal features of this cell death are different from those observed in mice lacking neurotrophins or neurotrophin receptors^{7,8}. □

Methods

Generation of *erbB3* mutant mice. Genomic *erbB3* DNA isolated from a library of 129 mouse DNA (isogenic to ES cell line E14) was used to construct the targeting vectors. A 5-kb fragment containing 7 exons (corresponding to nucleotides 1,308–2,112 in the cDNA; see ref. 26 for numbering of the cDNA) of *erbB3* was replaced by either wild-type or mutant²⁷ *neo* genes to create *erbB3*^Δ targeting vectors. The vector containing the mutant *neo* was first introduced into E14-1 ES cells by electroporation; homologous recombination events were enriched by selection with low concentrations of G418 (0.2 mg ml⁻¹). Two independently mutated ES cell clones were identified. The second targeting vector containing wild-type *neo* was electroporated into heterozygous ES cells; homologous recombination events were enriched by selection with high concentrations of G418 (1 mg ml⁻¹). Two independent *erbB3*^Δ/*erbB3*^Δ cell clones were identified. To generate the *erbB3*⁻ allele by homologous recombination, a targeting vector was produced that contained the wild-type *neo* gene directly fused to an exon; the fusion point corresponds to nucleotide 1,342 in *erbB3* cDNA. Thus, termination codons present in the *neo* cassette were directly fused to coding sequences. In addition, 4.7 kb of genomic DNA containing exon sequences that correspond to 1,343–2,112 in the cDNA were deleted. The structure of the mutant loci and absence of additional integration was verified by Southern-blot hybridization on ES cell DNA. Chimaeras were produced by injection of mutant ES cells into C57BL/6 blastocysts. Mouse strains carrying the *erbB3*^Δ or *erbB3*⁻ mutation were established from mutant ES cells (two independent cell clones for each allele). Phenotypes were analysed on a C57BL/6/129 hybrid background and verified on a 129 background. Survival frequencies of *erbB3*^Δ/*erbB3*^Δ embryos did not depend on the genetic background.

Protein analysis. For western-blot analysis, total embryonic extracts were immunoprecipitated with antibodies against a C-terminal *ErbB3* peptide (Santa Cruz). Immunoprecipitates were run on SDS–polyacrylamide cells, transferred to nitrocellulose, and *ErbB3* protein was detected with anti-C-terminal *ErbB3* antibody using the ECL western-blotting detection system (Amersham).

GPI isoenzyme ratios were determined in extracts generated from tissues from E18 chimaeric embryos. GPI-1^a is expressed in 129 cells and thus in cells derived from ES cells; GPI-1^b is expressed in C57/BL6 and thus in cells derived from blastocysts¹⁵. For the analysis, highly chimaeric animals (>60%) were used. After separating GPI-1^a and GPI-1^b isoenzymes in tissue extracts by electrophoresis, isoenzyme ratios were determined using the NIH image-1.60 program. Tissues were tested from at least 2 individual animals obtained by injection of independent homozygous ES cell clones. To isolate Schwann-cell precursors, dorsal root ganglia of E12.5 embryos were explanted and grown for 12 days in a defined medium used previously for embryonic mouse neurons²⁸;

under these conditions Schwann-cell precursors migrate out of the explant and adhere to the culture dish. Ganglia containing the neuronal cells were removed after 11 days; the GPI ratio of extracts of the remaining Schwann-cell precursors was determined.

Microscopical analysis and *in situ* hybridization. Whole embryos or segments of the spinal cord containing L4 and L5 dorsal root ganglia were embedded in Technovit 7100 (Heraeus Kulzer) after fixation in 4% paraformaldehyde. Serial sections of 4–6 μ m through L4 and L5 dorsal ganglia were counterstained using toluidine blue; cells with a clear nucleus and nucleoli were counted in every sixth section²⁹. Counts were not corrected for double or split nucleoli and therefore the numbers of neurons determined at different developmental stages are not strictly comparable. The reduction in neuronal numbers in *erbB3* mutant embryos is probably an underestimate, because the average size of the remaining neurons was larger in mutant than in control embryos (data not shown).

A spinal cord segment just caudal to the entry point of the L4 ventral root was removed, fixed in 2.5% glutaraldehyde, postfixed with OsO₄, contrasted with 0.1% tannic acid and 2% uranyl acetate. After embedding in Epon 812, semithin sections from the rostral face were prepared, counterstained with toluidine blue and used to identify ventral roots. Ultrathin sections were prepared for electron microscopy. Numbers of ventral root axons were determined from electron micrographs at a 6,400-fold magnification; wild-type numbers were equivalent to those reported previously³⁰.

Immunofluorescence analysis was done with the following antibodies: anti-S100 antibodies (Dako), monoclonal mouse anti NF-160 antibodies (Sigma), polyclonal rabbit anti-peripherin (from M.-M. Portier), rhodamine-coupled α -bungarotoxin (Molecular Probes) and Cy-3 anti-rabbit or Cy-2 anti-mouse IgG (Jackson ImmunoResearch Labs). Samples were visualized by confocal microscopy (Leica).

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Scrambler and *yotari* disrupt the *disabled* gene and produce a *reeler*-like phenotype in mice

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Formation of the mammalian brain requires choreographed migration of neurons to generate highly ordered laminar structures such as those in the cortices of the forebrain and the cerebellum. These processes are severely disrupted by mutations in *reeler*¹ which cause widespread misplacement of neurons and associated ataxia in *reeler* mice^{2,3}. Reelin is a large extracellular protein secreted by pioneer neurons that coordinates cell positioning during neurodevelopment^{1,4–8}. Two new autosomal recessive

mouse mutations, *scrambler*⁹ and *yotari*¹⁰ have been described that exhibit a phenotype identical to *reeler*^{9–11}. Here we report that *scrambler* and *yotari* arise from mutations in *mdab1* (ref. 12), a mouse gene related to the *Drosophila* gene *disabled* (*dab*)¹³. Both *scrambler* and *yotari* mice express mutated forms of *mdab1* messenger RNA and little or no mDab1 protein. mDab1 is a phosphoprotein that appears to function as an intracellular adaptor in protein kinase pathways. Expression analysis indicates that *mdab1* is expressed in neuronal populations exposed to Reelin. The similar phenotypes of *reeler*, *scrambler*, *yotari* and *mdab1* null mice¹⁴ indicate that Reelin and mDab1 function as signalling molecules that regulate cell positioning in the developing brain.

The *scrambler* mutant mouse (*scm*) arose as a spontaneous autosomal recessive mutation with a phenotype similar to *reeler* (*rl*)^{9,11}, whereas the *yotari* mutation (*yot*) appeared during the generation of mice carrying a disruption of the gene encoding the receptor for inositol-1,4,5-trisphosphate¹⁰. Like *reeler* mice³, *scrambler* and *yotari* mice show an abnormal pattern of cerebral cortical lamination and a reduction in cerebellar size, accompanied by a lack of foliation and neural cell ectopia. However, neither of these mutations are alleles of *rl* and they do not affect the expression of *reelin* mRNA or protein^{10,11} (Fig. 1a). As Reelin is secreted normally in *scrambler* mice, it was suggested that *scm* may function downstream of Reelin in a signalling pathway that controls neuronal migration¹¹. To identify the *scm* gene, we established an intraspecific cross between *scrambler* mice (C3HeB/FeJLe background) and C57BL/6J mice. Consistent with previous findings⁹, we found no recombination between *scm* and the simple sequence repeat (SSR) marker D4Mit176 on chromosome 4 (Fig. 1b). Furthermore, we did not detect any recombination with D4Mit155, although we did detect recombination with the nearby markers D4Mit219 and D4Mit304 (Fig. 1b). These findings indicate that the *scm* gene is located near D4Mit176 and D4Mit155. Notable genes in this region include the leptin receptor (*Lepr*)¹⁵ and adenylate kinase 3 (*Ak3*) (Mouse Genome Database: www.informatics.jax.org/mgd.html).

To identify candidate genes in the *scrambler* region, several yeast artificial chromosome (YAC) and bacterial artificial chromosome (BAC) clones were selected that had either been mapped to the region during the course of the identification of the *diabetes* (*db*) mutation, a mutation of *Lepr*^{16,17}, or that had been reported to contain one or more of the Mit markers close to D4Mit176 and D4Mit155 (Whitehead mouse genome database: www-genome.wi).

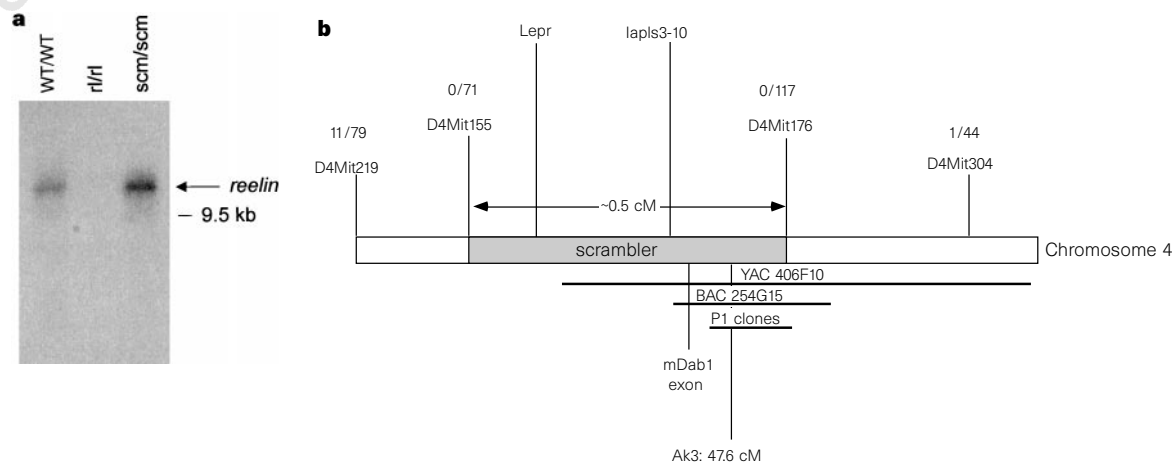


Figure 1 *Reelin* is expressed normally in *scrambler*. **a**, Northern analysis of 10 μ g of total brain RNA from wild-type, *reeler* and *scrambler* mice with a *reelin* probe. Map of the *scrambler* genomic locus. **b**, The *scrambler* locus maps to a region of chromosome 4 near the D4Mit155 and D4Mit176 markers, which showed no recombination with the mutant phenotype in 71 and 248 meioses, respectively. Markers outside the locus on either side (D4Mit219 and D4Mit304) showed 11 and

1 recombinations, respectively, with the *scrambler* phenotype in 71 meioses. Also indicated are the relative positions of the *Lepr*, *Ak3* and *mdab1* genes. YAC403F6 (MIT Whitehead database) and BAC254G15 (*db* contig¹⁶) contain D4Mit176 as well as an *mdab1* exon, as determined by PCR analysis. Three P1 clones (Genome Systems) contain D4Mit176 as well as the *Ak3* gene. *lpls3-10* is an IAP that shows no recombination with *mdab1* (0 recombinants in 93 mice).

mit.edu/cgi-bin/mouse/index). We demonstrated that two of these, YAC 406F10 and BAC 254G15, contained D4Mit176 (Fig. 1b). We also isolated three clones from a mouse P1 genomic library (Genome Systems) containing D4Mit176 and demonstrated that they contained portions of the Ak3 gene by exon trapping analysis.

Several genes that mapped to this region were evaluated as possible candidates for the *scrambler* gene. We focused on one particular candidate, *mdab1* (mouse disabled-1), a murine homologue of the *Drosophila* disabled gene (*dab*) which mapped close to *Lepr*¹², because it is expressed almost exclusively in the brain during development and because disruption of *mdab1* causes a phenotype similar to *reeler*¹⁴. Polymerase chain reaction (PCR) analysis revealed that at least one exon of *mdab1* is present in YAC 406F10, demonstrating close proximity to D4Mit176 (Fig. 1b).

The *scrambler* mutation resulted in barely detectable amounts of two *mdab1* mRNA species, the normal 5.5-kilobase (kb) mRNA, and an aberrant transcript of ~7 kb (Fig. 2a). In contrast, *yotari* mice expressed a shorter *mdab1* transcript at similar levels to the wild-type counterpart. Reverse transcriptase(RT)-PCR analysis revealed that the region between nucleotides 331 and 1,123 was altered in both *scrambler* and *yotari* (Fig. 2b, c). In *scrambler*, low levels of the normal product were detected, as well as a larger form consistent with the 7-kb RNA species detected by northern hybridization (Fig. 2a). In *yotari*, a smaller RT-PCR product was detected, consistent with the smaller mRNA described in Fig. 2a. Primers outside this region (19 to 447 and 1,091 to 2,116) amplified normalized products in both *scrambler* and *yotari*, suggesting that the mutations occur in the phosphotyrosine-interaction/phosphotyrosine-binding-domain (PI/PTB) of *mdab1* (ref. 12).

Nucleotide sequence analysis revealed that the larger *scrambler* transcript contains a 1,679-nucleotide insertion at position 570, whereas the smaller product is identical to the normal *mdab1* mRNA. A search of Genbank showed that this region is a mouse

intracisternal-A particle (IAP) element. IAPs are reiterated transposable elements, closely related to retroviruses, which have been associated with other mutagenic events that affect mRNA synthesis¹⁸. An IAP (Iapls3-10) was previously mapped to this region of the wild-type mouse genome and was shown not to recombine with *mdab1* in an interspecific backcross panel of 93 mice (Jackson BSS panel: (C57BL/6J × SPRET/Ei)F1 × SPRET/Ei)^{12,19} (Fig. 1b). The presence of the IAP in the large *scrambler* transcript introduces multiple stop codons that truncate the mDab1 open reading frame in the PI/PTB domain. Taken together, the genetic mapping data and RT-PCR results indicate that the IAP sequence is present in an intron of the *mdab1* gene. In *yotari*, nucleotide sequence analysis revealed that the *mdab1* mRNA transcript is also altered at position 570. However, in this case, the disruption results in the loss of 357 bases of coding sequence, a deletion that is predicted to maintain the reading frame of the encoded protein. Thus, the *scrambler* and *yotari* mutations alter *mdab1* splicing in the vicinity of an IAP element.

In both *scrambler* and *yotari*, *mdab1* mRNA species are present that might encode protein products, so we used polyclonal antibodies against regions of mDab1 for immunoblotting studies. As shown in Fig. 3, antibodies specific for the PI/PTB domain readily identified the 80K mDab1 protein in wild-type and *reeler* brain extracts. In contrast, this protein was drastically reduced in extracts prepared from *scrambler* brain and it was not detected in *yotari* extracts (Fig. 3). In *scrambler*, we identified approximately 5% of the wild-type level of mDab1 protein, consistent with the small amounts of normal-sized mRNA detected by northern analysis. Using additional antisera directed against peptides in the N- and C-terminal regions of mDab1, we were unable to detect any novel mDab1 polypeptides in *scrambler* or *yotari* extracts. Thus, if the mutant transcripts generate any mDab1 polypeptides, they must be rapidly degraded.

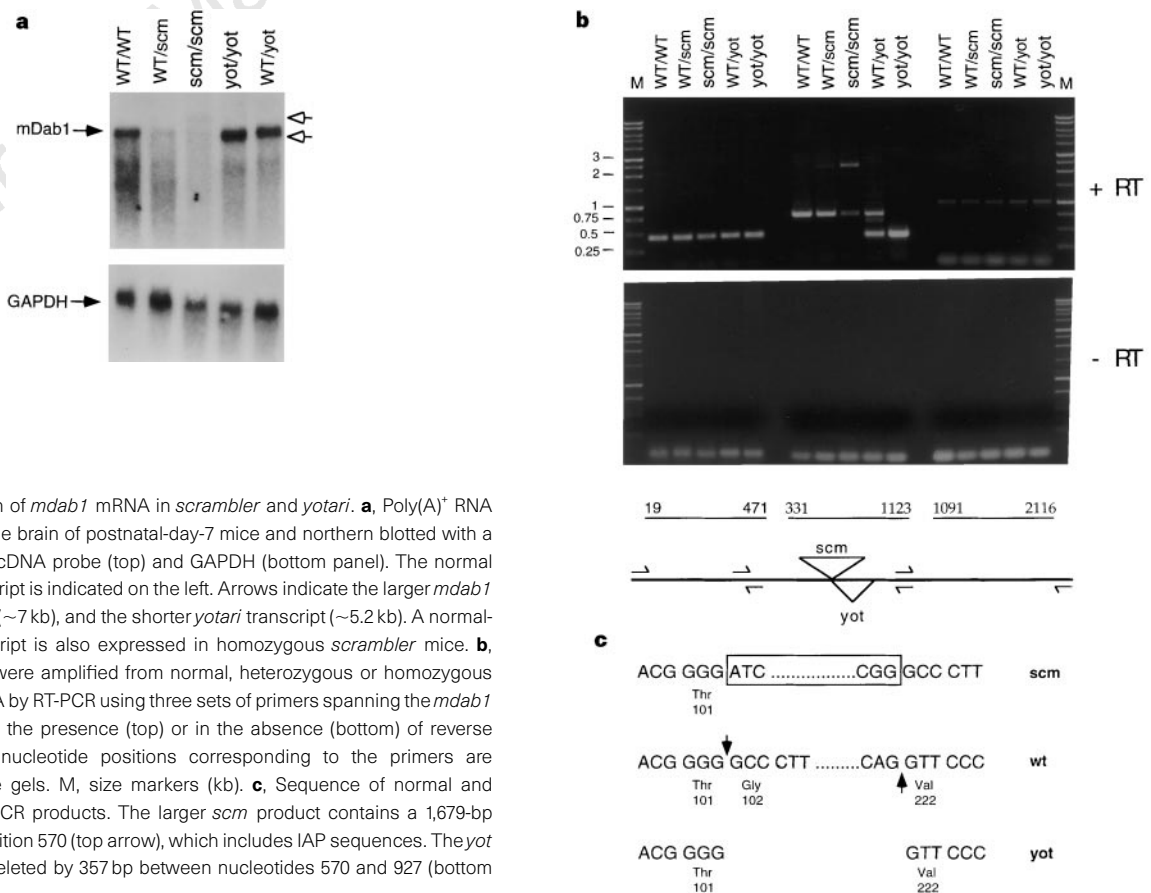


Figure 2 Expression of *mdab1* mRNA in *scrambler* and *yotari*. **a**, Poly(A)⁺ RNA was isolated from the brain of postnatal-day-7 mice and northern blotted with a ³²P-labelled *mdab1* cDNA probe (top) and GAPDH (bottom panel). The normal 5.5-kb *mdab1* transcript is indicated on the left. Arrows indicate the larger *mdab1* mRNA in *scrambler* (~7 kb), and the shorter *yotari* transcript (~5.2 kb). A normal-sized *mdab1* transcript is also expressed in homozygous *scrambler* mice. **b**, Regions of *mdab1* were amplified from normal, heterozygous or homozygous *scm* or *yot* brain RNA by RT-PCR using three sets of primers spanning the *mdab1* coding sequence in the presence (top) or in the absence (bottom) of reverse transcriptase. The nucleotide positions corresponding to the primers are indicated below the gels. M, size markers (kb). **c**, Sequence of normal and mutant *mdab1* RT-PCR products. The larger *scm* product contains a 1,679-bp insert (boxed) at position 570 (top arrow), which includes IAP sequences. The *yot* mutant product is deleted by 357 bp between nucleotides 570 and 927 (bottom arrow).

A comparison of the related phenotypes of the *reeler*, *scrambler*, *yotari* and *mdab1* null mice suggests that mDab1, like Reelin, regulates neuronal cell positioning. To compare the distribution of expression of *mdab1* and *reelin*, we did *in situ* hybridization analysis on embryonic-day-13.5 (E13.5) mouse brain tissue. *mdab1* was expressed throughout the developing brain, at high levels in the cortex, particularly in the developing cortical plate (Fig. 4). It was also present at high levels in the differentiating zone of the developing cerebellum which contains premigratory Purkinje cells (Fig. 4). The distribution of *reelin* in the marginal zone of the cortex and in the superficial region of the cerebellum (external granule layer and transitory zone) is juxtaposed to that of *mdab1* (Fig. 4). This pattern of expression corresponds to the location of predicted Reelin cell targets^{1,8}. As Reelin is an extracellular protein⁴, these findings suggest that *mdab1*-expressing cells are exposed to Reelin at the time and location in which developmental abnormalities are discernible in *reeler* mice²⁰. In the cortex, this occurs as migrating neurons reach the marginal zone and form distinct layers in the cortical plate, and in the cerebellum it occurs as Purkinje cells migrate towards the external granule layer to form the Purkinje cell plate which is absent in *reeler*.

Together with findings described elsewhere in this issue¹⁴, which show that disruption of the *mdab1* gene causes a *reeler*-like phenotype, our results demonstrate that the *scrambler* and *yotari* phenotypes result from a loss of function of *mdab1*. *Dab* was uncovered during a screen for dosage-sensitive modifiers of the *Drosophila* homologue of the Abelson tyrosine kinase gene (*d-Abl*)¹³. Mutations in *dab* exacerbate the defects in axogenesis associated with the *abl*^{-/-} phenotype in flies. In an *abl*^{-/-} background, heterozygous mutations in *dab* disrupt axonal organization and embryos die at early larval stages. *mdab1* was identified in a yeast two-hybrid screen as a Src-binding protein¹². It can associate with several tyrosine phosphoproteins, including the Abl and Fyn tyrosine kinases, and it is phosphorylated on tyrosine during neurodevelopment¹². Phosphorylation of a related gene, *p96*, also referred to as mDab2, is increased after treatment of macrophages with colony-stimulating factor 1 (ref. 21). The similarity between the PTB domains of mDab1 and that of the adaptor protein Shc^{12,22} raises the possibility that mDab1 functions as an adaptor molecule in a tyrosine kinase signalling cascade required for the regulation of neuronal migration during brain development. Thus, it is possible that Reelin activates a tyrosine-phosphorylation pathway, either directly by association with specific receptors, or indirectly by binding to other extracellular ligands in cells that express mDab1. Alternatively, both Reelin

and mDab1 may be required for appropriate cell positioning during neurodevelopment, but they may function through independent mechanisms. In any event, the absence of Reelin or mDab1 results in a failure to respond to positional cues by the neuronal populations that go astray in the mutant mice.

Targeted disruption of two other genes have been reported to cause defects that resemble *reeler*. Disruption of the cyclin-dependent kinase-5 gene (*cdk5*)²³ or its subunit *p35* (ref. 24) results in defective layer formation in the cerebral cortex. *cdk5* null mice resemble *reeler* in several regions of the brain, but unlike *reeler* mice, they die shortly after birth. In contrast, *p35* null mice are not ataxic and they have no obvious *reeler*-like defects in the hippocampus and cerebellum. It is likely that other subunits, such as *p39*, function as activating subunits of Cdk5 in these regions of the brain²⁵. Although some aspects of these mutants differ significantly from *reeler* and *scrambler*, it is possible that the Cdk5/p35 protein kinase complex participates in a common signalling pathway with Reelin and mDab1 in some neuronal populations.

Dab is essential for proper axogenesis in flies, particularly in the presence of Abl (ref. 13). Reelin is required for maturation and branching of axons of the entorhinohippocampal projections²⁶. Furthermore, mDab1 has been localized to growing nerves in embryonic mice¹². Thus, both mDab1 and Reelin may contribute to the elaboration of axons in mammals. It will now be interesting

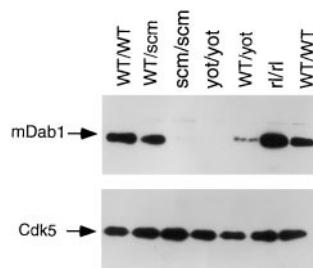


Figure 3 Analysis of mDab1 protein expression. Brain extracts from mice of the indicated genotype were assayed for mDab1 protein expression by western blot analysis using an antibody directed against the PTB domain of mDab1 (top). mDab1 protein (~80K) was detected in normal mice (WT/WT) and in heterozygous *scm* or *yot* mice, although at reduced levels. In homozygous *scrambler* mice, a residual mDab1 protein was present, whereas in homozygous *yotari* the protein was undetectable. Homozygous *reeler* mice expressed slightly higher levels of mDab1 compared to normal littermates (WT/WT sample, right). Equal amounts of the same samples were probed with an antibody directed against Cdk5 (bottom).

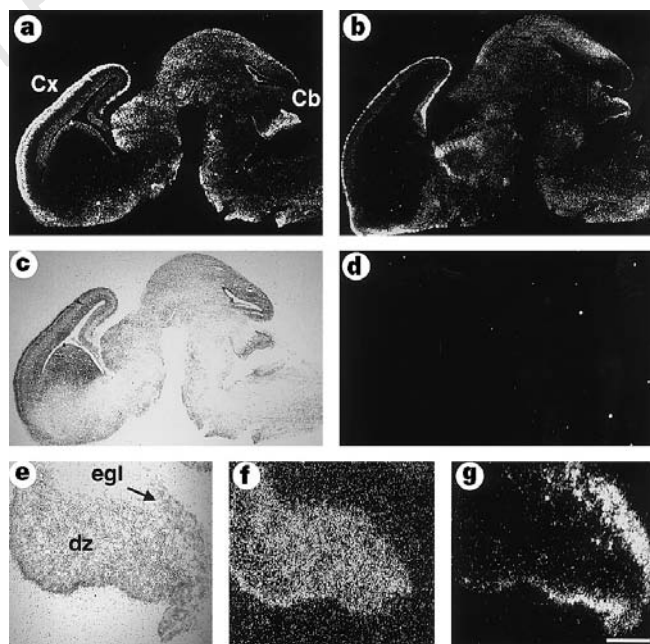


Figure 4 Distribution of *mdab1* and *reelin* mRNA in E13.5 mouse brain sagittal sections. **a**, Localization of *mdab1* with an antisense probe. The darkfield image shows dense labelling in discrete regions of the developing cortex (Cx), cerebellum (Cb), and other areas of the brain. **b**, Distribution of *reelin* mRNA, visualized with an antisense probe, on a neighbouring section. *reelin* mRNA was found in more restricted locations in the developing cortex and cerebellum. **c**, Brightfield image of the section shown in **a**. **d**, Darkfield view of a similar section hybridized with a *mdab1* sense probe. **e-g**, Localization of *mdab1* and *reelin* mRNA in E13.5 cerebellum. **e**, Brightfield view of the developing cerebellum in a sagittal section. At this age, the external granule layer (egl; arrow) and differentiating zone (dz) are discernible. The external granule layer gives rise to granule cells, whereas Purkinje cells arise from the differentiating zone. **f**, A darkfield view of the same section hybridized with a *mdab1* antisense probe shows that cells in the differentiating zone express *mdab1*. **g**, *reelin* mRNA distribution in an adjacent section. *reelin* mRNA is expressed in cells of the external granule layer (arrow) and the transitory zone adjacent to *mdab1* expressing cells in the differentiating zone. Scale bar: ~800 μ m in **a-d**; 100 μ m in **e-g**.

to investigate whether other mouse homologues of fly modifiers of Abl, such as *prospero*^{27,28} and *ena*^{29,30}, are involved in Reelin function. □

Methods

Mouse crosses and PCR mapping. Genetic mapping was performed in a cross between *scrambler* (C3HeB/FeJLe background) and C57BL/6J mice. DNA was extracted from tissues isolated from F₂ progeny and genotyped by PCR analysis. 500 ng of genomic DNA was amplified by PCR as described³¹. Reaction products were electrophoresed on 8–10% denaturing PAGE, the gels were dried and exposed to Kodak X-OMAT film for 8–16 h at room temperature. The exon of *mdab1* present in YAC403F6 and BAC254G15 was amplified using two primers: exon 1, (GTCAGGATCGCAGCGAAGCCACTTTG); and exon 2, (CCTTGAGCTTCATCATGGAATCTTG), which amplify the region between 331 and 471 (positions relative to the first nucleotide of the mDab1 Genbank file, accession number Y08379).

Northern blot hybridization. Total RNA was isolated from brains of postnatal-day-14 mice and poly(A)⁺ mRNA was prepared from postnatal-day-7 mice using an Invitrogen kit following the manufacturer's instructions. 10 µg of total or 3 µg poly(A)⁺ mRNA was loaded per lane on a 1.0% agarose-formaldehyde gel and hybridized with a full-length *mdab1* or *reelin* cDNA probe as described¹². The same blots were reprobed with riboprobes synthesized from the pTRI-GAPDH template (Ambion) using T7 RNA polymerase (Promega).

RT-PCR analysis. 100 ng poly(A)⁺ RNA isolated as described was reverse-transcribed and 1:20 of each RT reaction was subjected to RT-PCR analysis with the Takara LA-PCR kit using cycle conditions recommended by the manufacturer. Three PCR primer pairs spanning the coding region of *mdab1* were used. The starting and ending bases of each primer pair are as follows, with the forward and reverse primers in each pair, respectively, listed in 5' to 3' orientation. Primer pair 1: 19–48, 471–447; primer pair 2: 331–356, 1,123–1,091; primer pair 3: 1,091–1,123, 2,116–2,092.

Brain extract preparation and immunoblot analysis. Protein extracts of mouse brains were prepared by Dounce-homogenizing brains snap-frozen in liquid nitrogen in 500 µl ice-cold lysis buffer (0.1% N-P40, 250 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM PMSF, 20 µM leupeptin, 50 mM NaF) per 100 mg tissue. Extracts were cleared by microcentrifugation at 14,000 r.p.m. for 30 min. 100 µg protein extract was loaded per lane of a 4–12% polyacrylamide gradient gel (Novagen), electrotransferred to nitrocellulose membranes, incubated with a rabbit polyclonal antibody directed against the PTB domain of mDab1 (antibody B3; ref. 12), or with an anti-Cdk5 antibody (Santa Cruz), and visualized by enhanced chemiluminescence (ECL, Boehringer Mannheim).

In situ hybridization. Timed pregnant females were killed by cervical dislocation. Embryos were fixed overnight in 4% paraformaldehyde (in 0.1M phosphate buffer; pH 7.2), cryoprotected with 20% sucrose in buffer, embedded, and sectioned (10 µm). Radioactive *mdab1* (nucleotides 1,935–2,116) and *reelin* (nucleotides 5,818–5,973) sense and antisense probes were generated by *in vitro* transcription using ³³P-UTP. Hybridization was done overnight at 60 °C in a humid chamber as described¹. After washing, sections were counterstained with 0.1% toluidine blue and coverslipped.

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Neuronal position in the developing brain is regulated by *mouse disabled-1*

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During mammalian brain development, immature neurons migrate radially from the neuroectoderm to defined locations, giving rise to characteristic cell layers^{1,2}. Here we show that targeted disruption of the *mouse disabled1* (*mdab1*) gene³ disturbs neuronal layering in the cerebral cortex, hippocampus and cerebellum. The gene encodes a cytoplasmic protein, mDab1 p80, which is expressed and tyrosine-phosphorylated in the developing nervous system⁴. It is likely to be an adaptor protein, docking to others through its phosphotyrosine residues and protein-interacting domain⁴. The *mdab1* mutant phenotype is very similar to that of the *reeler* mouse^{5–7}. The product of the *reeler* gene, Reelin,

is a secreted protein that has been proposed to act as an extracellular signpost for migrating neurons⁸⁻¹⁰. Because mDab1 is expressed in wild-type cortical neurons, and Reelin expression is normal in *mdab1* mutants, mDab1 may be part of a Reelin-regulated or parallel pathway that controls the final positioning of neurons.

To determine the function of mDab1 p80, we made a targeted disruption of the first 47 codons of the protein-interacting (PI/PTB) domain (Fig. 1a). Mice heterozygous for the altered allele (*mdab1*-1) were generated by standard blastocyst manipulation and mouse breeding (Fig. 1b). In over 200 births, homozygous *mdab1*-1 mutants were born with the expected frequency. Western blot analysis of brain lysates showed that the mDab1 p80 protein is absent in the homozygotes (Fig. 1c).

The *mdab1*-1 homozygotes seem normal until 10 days post-partum (P10). By P15, it is apparent that *mdab1*-1 homozygotes are ataxic: they tremble, walk with a wide gait, drag their hind limbs, and frequently flip onto their backs. The mice generally die between P20 and P30.

The brains of *mdab1*-1 mutant mice have multiple defects when studied at P25 (Fig. 2, and data not shown). The cerebral cortex of the *mdab1*-1 mutant (Fig. 2b) lacks the distinct cell layers of the wild type (Fig. 2a). The cell-poor layer (marginal zone) under the pial (outer) surface is infiltrated by neurons in the mutant. In addition, fibre bundles are detected running close to the pial surface, suggesting that afferent fibres are running obliquely instead of radially through the cortex¹¹. The hippocampus and the dentate gyrus in the *mdab1*-1 mice are also indistinct. Normally, large

pyramidal neurons form discrete layers marking the dentate gyrus and CA1 and CA3 regions of the hippocampus (Fig. 2c), but in the mutant the large pyramidal neurons are dispersed, although vestiges of the normal structures are visible (Fig. 2d).

Cerebellar development is also severely affected. A normal cerebellum at birth consists of a sheet of Purkinje cells several cells thick covered by a superficial external granular layer. Starting at about P5, the Purkinje cells disperse to form a monolayer and the granule cells of the external granular layer proliferate and migrate inwards to form the inner granule layer. By P25, the normal cerebellum has an outer, cell-poor layer (the molecular layer), a single layer of Purkinje cells with dendritic arbors extending into the molecular layer, a broad inner granule layer, and an underlying layer of white matter (Fig. 2e). The mature cerebellum covers the midbrain. In contrast, the cerebellum of a *mdab1*-1 mutant at P25 is small and unfoliated, and the midbrain is exposed. The Purkinje cells are present in a

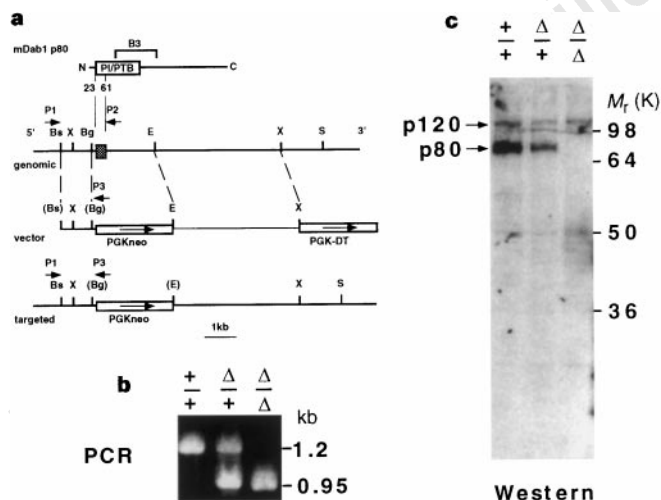


Figure 1 Disruption of the *mdab1* gene. **a**, Structure of the mDab1 p80 protein, with PI/PTB domain, binding site for B3 antibody, and the disrupted exon indicated. The knockout vector was designed with the phosphoglycerate kinase (PGK) promoter driving neomycin phosphotransferase in place of 2 kb of genomic sequences that contained the first exon of the PI/PTB domain. Homologous sequences of 0.9 kb (5') and 4 kb (3') flank the PGKneo cassette. Non-homologous integrants were counter-selected with the PGK-diphtheria toxin (DT) cassette. **b**, PCR genotyping of neonates. Oligonucleotides P1 and P2 hybridize to genomic sequences outside the homologous regions, as shown in **a**, and produce a 1.2-kb band by PCR amplification of the wild-type *mdab1* allele. Oligonucleotides P1 and P3 (hybridizing to the 5' end of the PGK promoter) amplify a 0.95-kb fragment from the *mdab1*-1 mutant allele. **c**, Western blot of brain lysate from neonate F₁ littermates probed with anti-mDab1 (B3) polyclonal antibody. mDab1 p80 is underexpressed in heterozygous animals and absent in the *mdab1*-1 homozygote. The 120K immunoreactive protein present in wild-type and mutant animals is expressed early in development but not in adults³, and is either a spliced *mdab1* gene product lacking the PI/PTB domain, or the product of a closely related gene.

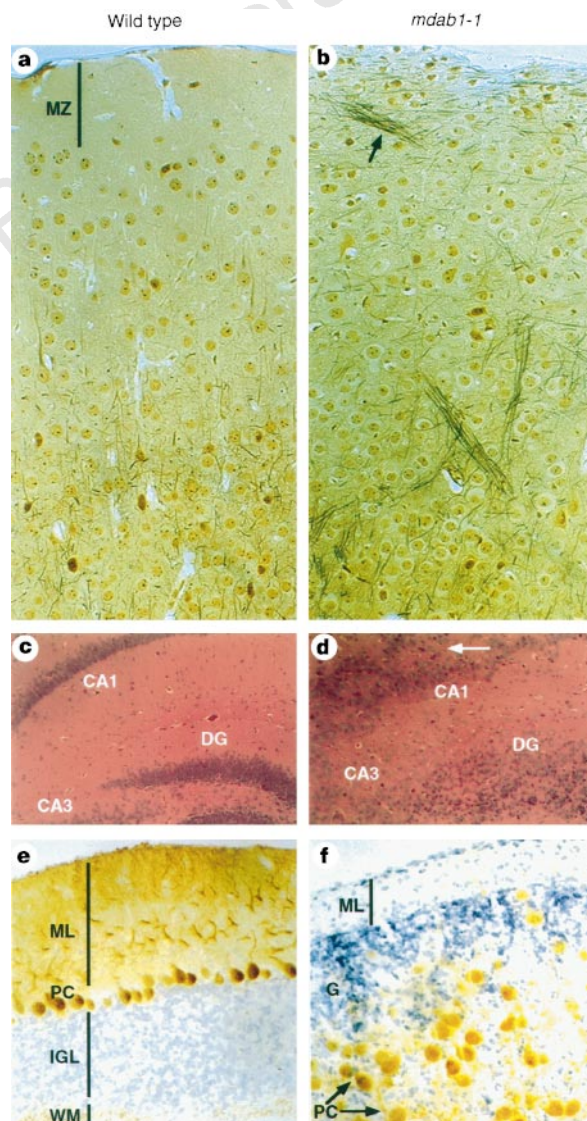


Figure 2 Alterations in the *mdab1*-1 mutant brain. Coronal sections of neocortex (Bielschowski stain) (**a**, **b**), hippocampus (haematoxylin and eosin stain) (**c**, **d**) and cerebellum (**e**, **f**) (brown, anti-Calbindin antibody; blue, Nissl stain) of wild-type (**a**, **c**, **e**) and *mdab1*-1 (**b**, **d**, **f**) mice. Arrows mark aberrant nerve fascicles (**b**) and split CA1 tract (**d**) in the mutant. In **f**, note that Nissl-stained granule cells are superficial to mislocalized calbindin-containing Purkinje cell bodies and dendritic arbors. Abbreviations: MZ, marginal zone; DG, dentate gyrus; ML, molecular layer; PC, Purkinje cells; G, granule cells; IGL, inner granule layer; WM, white matter.

central mass and their dendrites are oriented randomly (Fig. 2f), and they appear to have matured normally, because they express both calbindin (Fig. 2f) and zebrin II (ref. 12; data not shown). The granule cells are few in number and, although some migrate inwards, most remain superficial to the Purkinje cells (Fig. 2f). The anomalies in granule-cell proliferation and relative position may be secondary to the failure of the Purkinje cells to disperse into a monolayer¹¹.

In the neocortex, the position and fate of a neuron is strongly correlated with its birthdate². Birthdate studies have been done by labelling mice *in utero* with DNA precursors, and identifying labelled neurons in the cortex after birth¹³. Cells undergoing their last S phase during the labelling period retain the label in their DNA, whereas cells that continue to cycle dilute the label over time. To test whether cortical neurons migrate correctly in *mdab1-1* mutant mice, we treated mice *in utero* with 5-bromodeoxyuridine (BrdU) and analysed their brains at P25. Nuclei that were labelled with BrdU at embryonic day 11 (E11) were found deep in the cortex of wild-type animals (layer VI)¹³, but were superficial in the *mdab1-1* mutants (Fig. 3a, b). Conversely, neurons labelled at E16 were predominantly in the superficial cortex (layers II–III) of wild-type mice¹³, but remain deep in the cortex of *mdab1-1* mutant littermates (Fig. 3c, d). This shows that the final positions of cortical neurons are abnormal in *mdab1-1* mutant mice. Because large pyramidal cells, normally located deep in the cortex, are found near the surface of the mutant cortex (Fig. 2b, and data not shown), the abnormal layering of the mutant cortex may result from altered migration of neurons without an alteration in fate. Experiments with molecular markers will be needed to test whether cell fate is also altered.

These observations indicate that *mdab1-1* mutant mice resemble *reeler* mutants in neuroanatomy and neuron birthdates^{14–16}. Indeed, detailed histology of mature *mdab1-1* and *reeler* cerebella shows identical morphologies and the same abnormal compartmentation of Purkinje cells (data not shown). In both cases, the defects may be a consequence of altered neuronal migration. This suggests that the proteins mDab1 and Reelin could be involved in the same or parallel pathways that regulate neuronal migration. Reelin has been pro-

posed to serve as a marker for the locality at which radially migrating cortical neurons come to rest^{8,9}. Reelin is expressed by pioneer Cajal–Retzius neurons that occupy the cell-poor marginal zone of the neocortex. The *mdab1-1* phenotype raises the possibility that mDab1 might be needed either to make or to respond to the Reelin signal, or to convey another signal that acts in concert with the Reelin pathway.

mDab1 does not seem to be required for Cajal–Retzius cell migration or Reelin production, because indirect immunofluorescence shows that the Cajal–Retzius cells are present in the marginal zone and express Reelin in the *mdab1-1* neocortex at E16 (Fig. 4a, b). To test whether mDab1 might be in the cells responding to the Reelin signal, we localized mDab1 in the E16 cortex by immunofluorescence (Fig. 4c–e), and detected it in the cytoplasm of nearly all neurons in the developing cortical plate (Fig. 4c). mDab1 was found throughout the cortical plate, intermediate zone, and ventricular zone of the wild-type embryos (Fig. 4d). The reduced staining of mutant embryos (Fig. 4e) shows that staining is specific. Thus mDab1 is expressed by cortical neurons, consistent with it acting in response to external signals such as Reelin.

In the E16 wild-type but not mutant cerebellum, mDab1 was expressed in the cerebellar anlage, where the Purkinje cells are coalescing (Fig. 4f, g). Expression of mDab1 was detected in the external granular layer, and it is unlikely that Bergmann glia express mDab1 because they have cytoplasmic projections that would have been detected in the external granular layer. Because Purkinje cells in *mdab1-1* mutants are malpositioned at P0 before the start of granule cell ingress (data not shown), the primary defect in the mutant cerebellum may be the result of defects in the Purkinje cells. It has been shown previously that granule cells depend on adjacent Purkinje cells for trophic support¹⁷, and that granule cells make Reelin¹⁸. Because Reelin expression is not altered in the *mdab1-1* mutants, and because mDab1 is expressed by the affected cell types in the neocortex and cerebellum, it seems likely that mDab1 acts cell-autonomously.

mDab1 has no known catalytic activity, so it may function as a docking protein to link proteins together through its amino-terminal PI/PTB domain and tyrosine-phosphorylated motifs³.

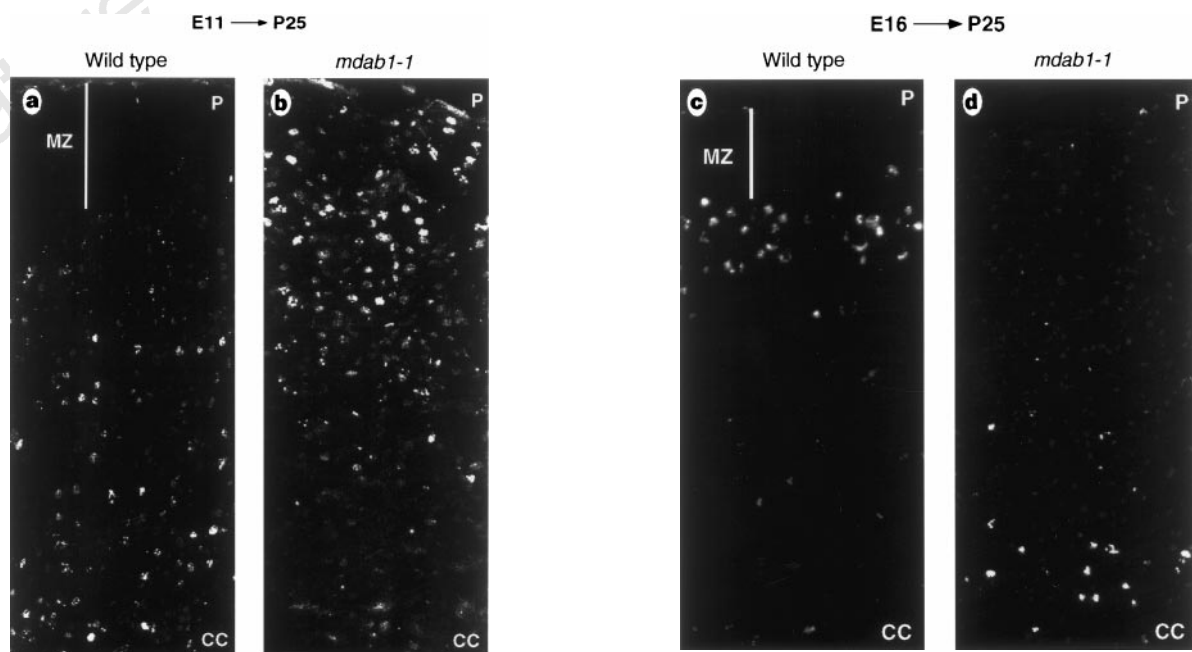


Figure 3 Altered positions of *mdab1-1* neurons in cerebral cortex. Animals were labelled with BrdU *in utero* at E11 (a, b) or E16 (c, d) and killed at P25. Coronal sections of cortices at the level of the hippocampus were prepared from wild-type

(a, c) and mutant (b, d) littermates, and immunostained with anti-BrdU antibodies (white). P, pia; CC, corpus callosum.

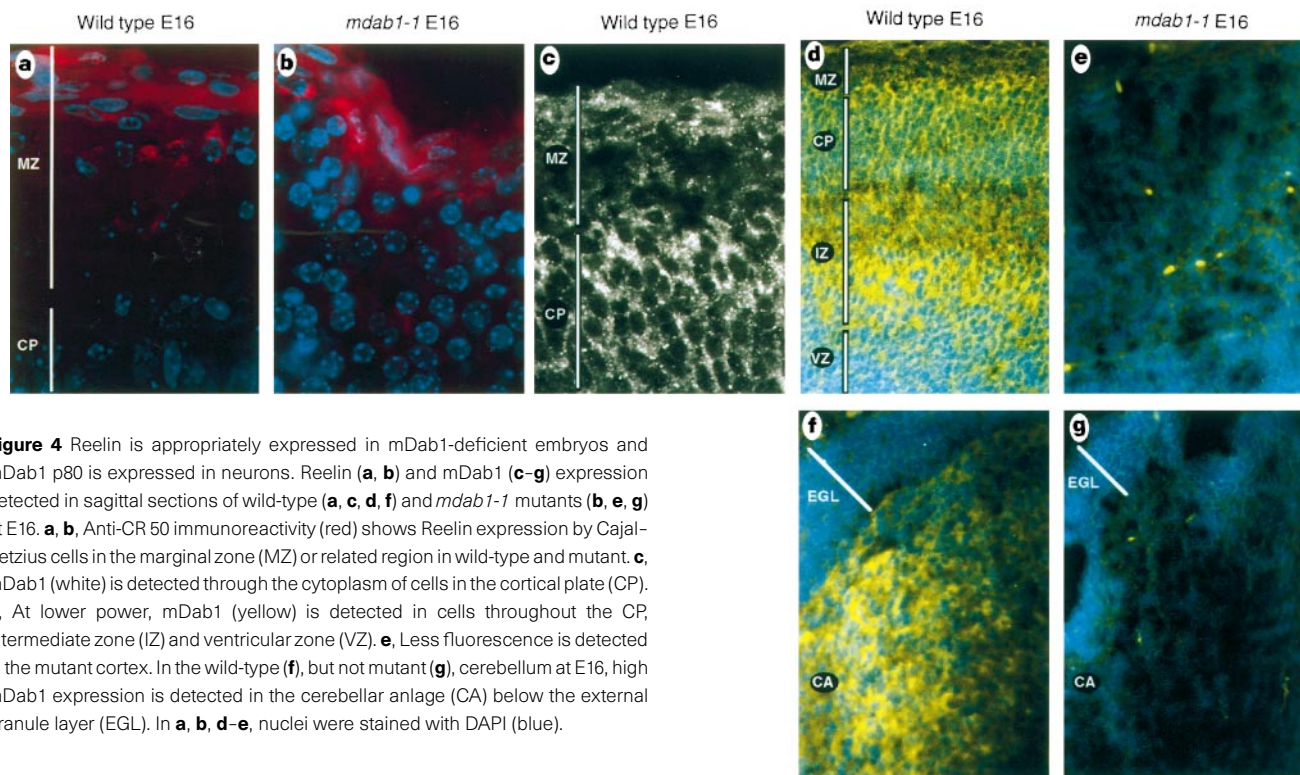


Figure 4 Reelin is appropriately expressed in *mdab1*-deficient embryos and *mDab1* p80 is expressed in neurons. Reelin (**a, b**) and *mDab1* (**c–g**) expression detected in sagittal sections of wild-type (**a, c, d, f**) and *mdab1-1* mutants (**b, e, g**) at E16. **a, b**, Anti-CR 50 immunoreactivity (red) shows Reelin expression by Cajal–Retzius cells in the marginal zone (MZ) or related region in wild-type and mutant. **c**, *mDab1* (white) is detected through the cytoplasm of cells in the cortical plate (CP). **d**, At lower power, *mDab1* (yellow) is detected in cells throughout the CP, intermediate zone (IZ) and ventricular zone (VZ). **e**, Less fluorescence is detected in the mutant cortex. In the wild-type (**f**), but not mutant (**g**), cerebellum at E16, high *mDab1* expression is detected in the cerebellar anlage (CA) below the external granule layer (EGL). In **a, b, d–e**, nuclei were stained with DAPI (blue).

This function may be regulated by extracellular signals. Thus proteins that modify and bind to *mDab1*, including non-receptor tyrosine kinases such as *Src* and *Abl*³, may regulate neuronal migration. Mutations in other components of the signalling pathway might be expected to cause a phenotype like that of *mdab1*. Mutations in *src* and *abl* do not affect brain development^{19–21}, but these tyrosine kinases may be redundant. Phenotypes resembling that of *mdab1-1* mutants are seen with mutations in *scrambler* (ref. 22), *yotari* (ref. 23), *Cdk5* (ref. 24) and *p35* (ref. 25); *scrambler* and *yotari* are alleles of *mdab1* (ref. 26). *Cdk5* and *p35* are the catalytic and regulatory subunits of a serine/threonine kinase that could potentially operate in a common signalling pathway with *mDab1* and Reelin. It remains possible, however, that these molecules operate on mechanistically distinct processes that control the laminar organization of neurons.

Disabled, the *Drosophila* homologue of *mdab1*, is required for axonal pathfinding or fasciculation, and acts together with *abl*^{27,28}. Redundant pathways regulate pathfinding²⁹, but it is not clear from this whether *Abl* and *Disabled* are on the same or parallel pathways. However, there are a number of parallels between axonal pathfinding and cell migration, and *Disabled*-like molecules may be involved in both processes. Pathfinding defects have not yet been observed in the *mdab1-1* mice. The *reeler* mutation affects some neuronal connections³⁰ but not others¹¹. Identification of *mDab1* binding partners and a receptor for Reelin will help us to understand how the activities of these proteins coordinate neuronal migration and axonal guidance. □

Methods

Production of mutant mice. The *mdab1-1* targeting vector was constructed in the PGKneox2DTA vector. A 0.9-kb *BseRI*–*BglI* fragment, which corresponds to intronic sequences 5' to the exon encoding residues 23–69 of the *mdab1* gene, was blunted and ligated into *NotI*-digested vector. A 4-kilobase *EcoRI*–*XbaI* fragment from the *mdab1* gene 3' to the same exon was linked and ligated 3' into the *Sall* site between the PGKneo and PGK-DT sequences producing p80KO1. AK7 embryonic stem (ES) cells (1×10^7) were electroporated with linearized p80KO1 (20 µg). Cell culture and blastocyst injections

were done as previously described¹⁹. Oligonucleotides used for PCR genotyping (Fig. 1) were P1 (5'-GTCAGGCTTCCTAAGTAGAAAGGA-3'), P2 (5'-TTCCAGGACGGAATCACTCAACC-3') and P3 (5'-GGGAAAAGCGCCTCCCC TACCCGGT-3'). Similar phenotypes were observed with two independently-derived ES clones, and in 129Sv congenic or C57Bl6/129Sv hybrid genetic backgrounds.

Histology and immunohistochemistry. Animals were fixed by perfusion with 4% paraformaldehyde in phosphate-buffered saline at 4 °C, except for anti-*mDab1* immunofluorescence studies in which a solution of dimethyl sulphoxide and methanol (1:4) was used. Haematoxylin and eosin, Nissl and Bielschowski stains were done following standard protocols. Anti-CR 50, anti-BrdU (Becton Dickinson) and anti-calbindin immunohistochemistry have been described previously^{9,25}. An antibody specific to *mDab1* p80 was generated by depleting anti-*mDab1* (B3) antibodies of reactivity to p120 by adsorption with lysates from *mdab1-1* mutant brains, and used essentially as described³. For birthdate analysis, BrdU was injected into pregnant mice (0.15 mg per g body weight) at the stage indicated¹⁶. Immunofluorescence images were collected using either Delta Vision (Applied Precision) or confocal microscopes (Fig. 4c; BioRad).

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A CD4⁺ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis

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Induction and maintenance of peripheral tolerance are important mechanisms to maintain the balance of the immune system. In addition to the deletion of T cells and their failure to respond in certain circumstances, active suppression mediated by T cells or T-cell factors has been proposed as a mechanism for maintaining peripheral tolerance¹. However, the inability to isolate and clone regulatory T cells involved in antigen-specific inhibition of immune responses has made it difficult to understand the

mechanisms underlying such active suppression. Here we show that chronic activation of both human and murine CD4⁺ T cells in the presence of interleukin (IL)-10 gives rise to CD4⁺ T-cell clones with low proliferative capacity, producing high levels of IL-10, low levels of IL-2 and no IL-4. These antigen-specific T-cell clones suppress the proliferation of CD4⁺ T cells in response to antigen, and prevent colitis induced in SCID mice by pathogenic CD4⁺CD45RB^{high} splenic T cells. Thus IL-10 drives the generation of a CD4⁺ T-cell subset, designated T regulatory cells 1 (Tr1), which suppresses antigen-specific immune responses and actively downregulates a pathological immune response *in vivo*.

Immune tolerance towards self antigens is dependent on the ability of the immune system to discriminate between self and non-self^{2,3}. This occurs mainly through clonal deletion in the thymus of self-reactive T lymphocytes at an early stage of development. However, the immune system is also exposed to extrathymic self antigens and to repetitive stimulation by non-pathogenic antigens through inhalation and ingestion of foreign substances. To avoid chronic cell activation and inflammation, the immune system must develop unresponsiveness to such stimuli. Several mechanisms of peripheral tolerance have been proposed, including T-cell anergy^{4,5}, T-cell deletion², and active immune suppression¹. Understanding these mechanisms of tolerance induction is clinically relevant for the treatment of autoimmune diseases and in transplantation, where the graft must ultimately be recognized as self.

The induction of anergy⁵ and cell deletion by apoptosis² have been documented both *in vitro* and *in vivo*, but the analysis of active immune suppression mediated by T cells has been hampered by the inability to generate and clone these cells *in vitro*. The importance of cytokines in the development of specialized Th cells is now clear. Th1 cells are induced by activation in the presence of IL-12, whereas IL-4 drives the differentiation of Th2 cells⁶. Here we show that repetitive stimulation of CD4⁺ T cells in the presence of IL-10 induces the differentiation of a unique subset of T cells with immunoregulatory properties.

Ovalbumin (OVA)-specific naive CD4⁺ T cells obtained from the αβ T-cell antigen receptor (TCR) DO11-10 transgenic mice⁷ repeatedly stimulated with splenic antigen-presenting cells (APCs) and OVA peptide in the presence of IL-10, or IL-4 and IL-10, displayed a cytokine profile distinct from that of the classical Th0, Th1 or Th2 phenotype⁸. These T cells produce high levels of IL-10 and IL-5 and low levels of IL-2 and IL-4 (Fig. 1). Another characteristic of these T-cell populations is that they proliferated poorly in response to antigenic stimulation (Fig. 2a). In contrast, CD4⁺ T cells isolated from OVA-specific TCR transgenic mice repeatedly stimulated with OVA peptide in the presence of IL-4 alone displayed the typical Th2-type profile, secreting IL-4, IL-5 and IL-10 (Fig. 1)⁹.

Analysis of T-cell clones isolated from the mouse CD4⁺ T-cell populations that were repeatedly stimulated with OVA peptide and splenic APCs in the presence of IL-10 showed that half of the CD4⁺ T-cell clones obtained displayed this cytokine profile. They produced high levels of IL-10 and undetectable levels of IL-2 and IL-4, whereas the levels of production of interferon (IFN)-γ and transforming growth factor (TGF)-β were comparable with those of Th0 and Th1 clones, respectively (Table 1). These T-cell clones also proliferated poorly in response to antigen-specific stimulation (Fig. 2a), which may explain the previous failure to isolate these cells. Only Th1, Th2 and Th0 clones were isolated from T-cell populations cultured in the absence of IL-10 (Table 1). Thus chronic activation of mouse CD4⁺ T cells in the presence of IL-10 results in the generation of a T-cell subset with a unique cytokine profile and low proliferative capacity. These cells are designated Tr1 cells on the basis of their function.

Tr1 cells can also be generated from human peripheral blood. Both alloantigen-specific CD4⁺ T-cell clones (JDV24) and non-alloantigen-specific T-cell clones (JDV15, JDV308 and JDV94)

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displaying the Tr1 cell cytokine profile were derived from CD4⁺ T-cell populations that had initially been stimulated with allogeneic monocytes in the presence of IL-10 (Table 2). In contrast, only Th1-type clones were obtained when the CD4⁺ T cells were stimulated by allogeneic monocytes in the absence of IL-10. The most striking characteristic of the human Tr1 cell clones was their unusually high level of IL-10 production (Table 2). They also produced very low levels of IL-2, and failed to secrete detectable levels of IL-4, whereas their levels of IL-5, IFN- γ and TGF- β production were similar to those of human Th0 clones. Taken together, these results demonstrate that antigen-specific Tr1 cells can be generated *in vitro* from naive populations of both human and mouse CD4⁺ T cells.

Human Tr1 clones stimulated with either immobilized anti-CD3 and anti-CD28 monoclonal antibodies or allogeneic monocytes (for

JDV24) showed low proliferative responses that were sustained by secretion of IL-2, as the addition of an anti-IL-2 monoclonal antibody completely blocked the growth of the Tr1 cells (Fig. 2b). Kinetic studies showed that IL-10 is produced rapidly after activation of the human Tr1 clones. IL-10 is detectable in the supernatants of Tr1 clones 8 h after activation (data not shown), which indicates that IL-10 production by Tr1 clones generally occurs before or at the same time as IL-2 production. In contrast, production of IL-10 by Th0, Th1 and Th2 clones established from the same donor in the same experiment was not detectable until 20 h after activation, which is compatible with previous findings¹⁰.

Early endogenous IL-10 production accounts in part for the low proliferative capacity of the human and mouse Tr1 clones in response to TCR/CD3 stimulation (Fig. 2), as proliferation of the

Table 1 Cytokine production by murine OVA-specific CD4⁺ T-cell clones

T-cell clones	Initial incubation with IL-10	IL-2 (pg ml ⁻¹)	IL-4 (pg ml ⁻¹)	IL-10 (pg ml ⁻¹)	IFN- γ (ng ml ⁻¹)	TGF- β (pg ml ⁻¹)	Clone type
A-10-1	yes	<40	<50	1,230 $\pm$ 310	105 $\pm$ 27		Tr1
A-10-9	yes	<40	<50	1,020 $\pm$ 120	87 $\pm$ 17	435 $\pm$ 9	Tr1
A-10-11	yes	<40	<50	1,850 $\pm$ 410	217 $\pm$ 68	747 $\pm$ 9	Tr1
A-10-14	yes	<40	<50	1,820 $\pm$ 120	198 $\pm$ 67		Tr1
A-10-2	yes	102 $\pm$ 26	250 $\pm$ 45	850 $\pm$ 130	489 $\pm$ 124		Th0
A-10-7	yes	97 $\pm$ 27	897 $\pm$ 102	410 $\pm$ 120	267 $\pm$ 247		Th0
A-10-15	yes	52 $\pm$ 19	89 $\pm$ 17	205 $\pm$ 80	420 $\pm$ 41		Th0
A-10-19	yes	69 $\pm$ 27	890 $\pm$ 48	310 $\pm$ 120	107 $\pm$ 57		Th0
A-3	no	<40	1,247 $\pm$ 157	510 $\pm$ 110	<75		Th2
A-5	no	<40	879 $\pm$ 87	310 $\pm$ 60	<75		Th2
A-7	no	129 $\pm$ 48	<50	<75	375 $\pm$ 97	715 $\pm$ 2	Th1
A-13	no	89 $\pm$ 28	598 $\pm$ 59	215 $\pm$ 30	143 $\pm$ 13		Th0
A-14	no	203 $\pm$ 49	<50	<75	249 $\pm$ 49		Th1

Cytokine production by murine OVA-specific CD4⁺ T-cell clones derived from T cells of DO11.10 TCR-transgenic mice repetitively stimulated in the presence or absence of IL-10. Naive (MEL14 bright) CD4⁺ T cells from DO11.10 transgenic mice were stimulated with OVA peptide (0.6 μ M) in the presence (A-10-) or absence (A-) of IL-10 (100 U ml⁻¹). After repetitive stimulations under the same conditions, the CD4⁺ T cells from these two populations were cloned by limiting dilution. After expansion, the T-cell clones were stimulated by OVA peptide (1 μ M) and irradiated total splenic APCs. Cytokines were analysed by ELISA in culture supernatants collected after 48 or 72 h of culture for TGF- β . Results represent pooled data from 4 representative experiments.

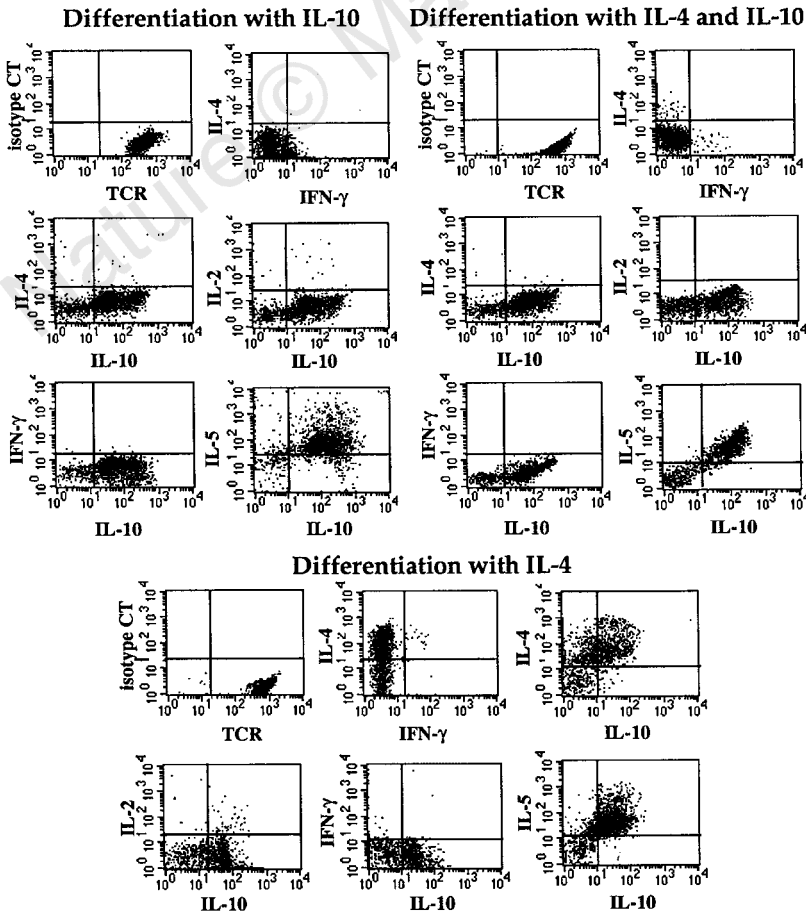


Figure 1 Generation of murine Tr1 cells in the presence of IL-10. Naive (MEL14 bright) CD4⁺ T cells from BALB/c mice transgenic for the DO11.10 $\alpha\beta$ TCR⁷ were cultured with OVA peptide (0.6 μ M) and irradiated splenic APCs in the presence or absence of: IL-4 (200 U ml⁻¹), IL-10 (100 U ml⁻¹), or both. The stimulation, under the same conditions, was repeated weekly for three consecutive weeks⁹. Cells were collected, washed and restimulated with immobilized anti-CD3 (10 μ g ml⁻¹) and anti-CD28 mAb (10 μ g ml⁻¹) for 4 h at 37 $^{\circ}$ C. Brefeldin A (10 μ g ml⁻¹) was added for the last 2 h of culture²⁹. The cells were fixed and stained for detection of intracellular cytokines using FITC or PE-conjugated monoclonal antibodies specific for IFN- γ , IL-2, IL-4, IL-5 and IL-10, or an isotype-matched control mAb (isotype CT), as indicated. Cells were also stained with the anti-TCR clonotype-specific mAb KJ1-26 (TCR) to show that all cells were positive for the transgene after 3 weeks of culture. One of three experiments is shown.

Tr1 clones was augmented by the addition of a neutralizing anti-IL-10 monoclonal antibody, which is consistent with previous observations indicating that IL-10 prevents or inhibits T-cell proliferation¹¹. In contrast, anti-IL-10 monoclonal antibody had no effect on the proliferative responses of control Th1, Th2 or Th0 clones (Fig. 2). Proliferation of both human and mouse Tr1 clones was also partly augmented by a neutralizing anti-TGF- β monoclonal antibody (Fig. 2), whereas the proliferation of the control Th clones was unaffected (Fig. 2). The combination of anti-IL-10 and anti-TGF- β antibodies had additive effects and almost completely restored the proliferative responses of Tr1 clones (Fig. 2).

The observation that Tr1 cells secrete high levels of the immunosuppressive cytokine IL-10, and low levels of the T-cell growth-promoting cytokines IL-2 and IL-4, suggested that antigen-specific activation of Tr1 cells may result in inhibition of antigen-specific proliferation of other T cells. Indeed, co-culture experiments using a transwell system confirmed this notion for both human and mouse T cells. Resting human CD4⁺ T cells were stimulated with irradiated allogeneic monocytes in the bottom compartment, whereas alloantigen-specific Tr1 clone JDV24 or non-antigen-specific Tr1 clone

JDV308 were stimulated with the same allogeneic monocytes in the top compartment. Alloantigen-specific stimulation of the Tr1 clone JDV24 strongly reduced the alloantigen-specific proliferative responses of autologous human CD4⁺ T cells, whereas the non-antigen-specific Tr1 clone JDV308 was ineffective (Fig. 3a). A slight increase in CD4⁺ T-cell proliferation was noticed following co-culture with the alloantigen-specific T-cell clone JDV305, which belongs to the Th1 subset (Fig. 3a).

Similar data were obtained with mouse Tr1 clones (Fig. 3b). The proliferation of naive CD4⁺ T cells in response to OVA peptide and splenic APCs was dramatically reduced following co-culture in the transwell system with OVA peptide-activated Tr1 clones or with activated Tr1-cell populations generated in the presence of IL-10. In contrast, OVA-specific Th0 clones or Th2-cell populations generated in

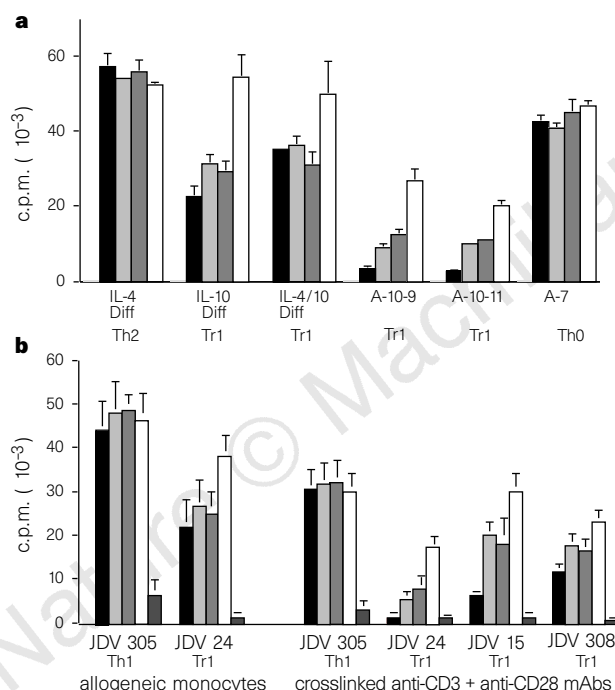


Figure 2 Proliferative responses of Tr1 cells. **a**, Naive (MEL14 bright) CD4⁺ T cells from BALB/c mice transgenic for the DO11.10 $\alpha\beta$ TCR⁷ were differentiated (Diff) *in vitro* as described in Fig. 1 and restimulated with OVA peptide (0.6 μ M) and irradiated splenic APCs for 3 days. One OVA-specific Th0 clone (A-7) and two OVA-specific Tr1 type clones (A-10-9) and A-10-11) were stimulated using the same conditions. **b**, The human Th1 (JDV 305) and Tr1 (JDV24) alloantigen-specific CD4⁺ T-cell clones or the non-antigen-specific Tr1-cell clones (JDV15 and JDV 308) were stimulated with either allogeneic irradiated monocytes for 5 days or with immobilized anti-CD3 (10 μ g ml⁻¹) and anti-CD28 mAb (1 μ g ml⁻¹) for 3 days. The proliferative responses were measured by ³[H]-TdR incorporation during the last 12 h of culture. Cultures of human or mouse T cells were carried out in the presence of an isotype-matched control mAb (GL113, rat IgG1 at 20 μ g ml⁻¹ + mouse IgG1 from Caltag (Burlingame, CA) at 20 μ g ml⁻¹; black bars); a neutralizing anti-IL-10 mAb (12G8 at 5 μ g ml⁻¹ for human T cells or JES5-2A5 at 10 μ g ml⁻¹ for mouse T cells; white hatched bars); a neutralizing anti-TGF- β (from Genzyme (Cambridge, MA) at 10 μ g ml⁻¹; black hatched bars); a combination of the two antibodies (white bars); or a neutralizing anti-IL-2 mAb (BG-5, 10 μ g ml⁻¹ for human cells; grey bars). A representative Th1 (JDV 305) and a representative Tr1 (JDV24) are shown for antigen-specific response. One of four experiments is shown.

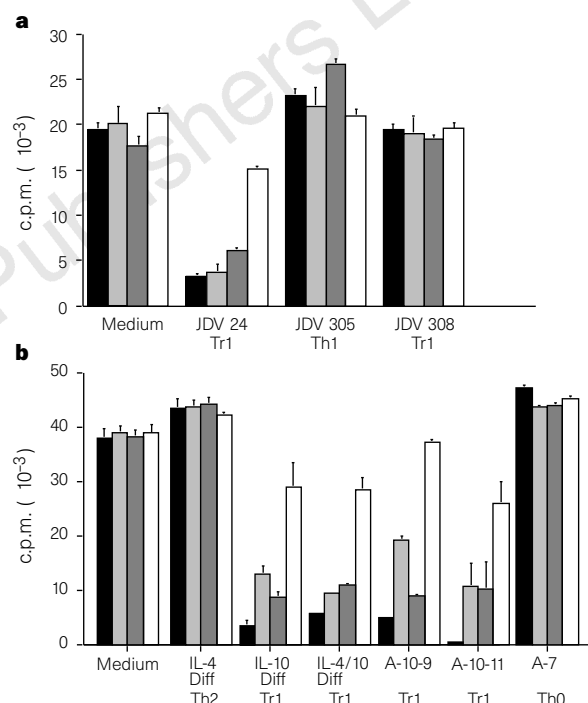


Figure 3 Tr1 cells inhibit the antigen-specific proliferative responses of naive CD4⁺ T cells. **a**, Purified human CD4⁺ T cells from donor JDV were stimulated by allogeneic purified irradiated monocytes for 5 days in the bottom compartment of a transwell system. The resting T cells were co-cultured with various autologous T-cell clones contained in the upper transwell compartment; the Tr1 clone JDV 24 and the Th1 clone JDV 305 are alloantigen-specific, whereas the Tr1 clone JDV 308 is non-antigen-specific. After 5 days the basket was removed and the proliferative response of the CD4⁺ T cells was analysed by ³[H]-TdR incorporation during the last 12 h of culture. **b**, Naive (MEL14 bright) CD4⁺ T cells from BALB/c mice transgenic for the DO11.10 $\alpha\beta$ TCR were stimulated in the bottom compartment of a transwell system with OVA peptide (0.6 μ M) and irradiated APCs for 3 days. These naive T cells were co-cultured with the indicated populations contained in the upper transwell basket: OVA-specific CD4⁺ T-cell populations differentiated (diff) *in vitro* by three consecutive restimulations in the presence of IL-4 (IL-4 Diff), IL-10 (IL-10 Diff) or IL-4 and IL-10 (IL4/10 Diff), respectively, or OVA-specific CD4⁺ T-cell clones A-10-9, A-10-11 (Tr1 type clones) or A-7 (Th0 type clone). After 3 days the basket was removed and the proliferative response of the naive CD4⁺ T cells was analysed by ³[H]-TdR incorporation during the last 12 h of the culture. Cultures of human and murine cells were carried out in the presence of an isotype-matched control (GL113, rat IgG1 at 20 μ g ml⁻¹ + mouse IgG1 from Caltag Laboratories (Burlingame, CA) at 20 μ g ml⁻¹; black bars); a blocking anti-IL-10 mAb (12G8 at 5 μ g ml⁻¹ for human cells or JES5-2A5 at 10 μ g ml⁻¹ for mouse cells; white hatched bars); a blocking anti-TGF- β (from Genzyme (Cambridge, MA) at 10 μ g ml⁻¹; black hatched bars); or a combination of the two antibodies (white bars). Results represent one of three experiments performed.

Table 2 Cytokine production by human Tr1 cells

T-cell clones	Clone type	IL-2	IL-4	IL-5	IL-10	IFN- γ	TGF- β
Cells preincubated with IL-10							
JDV15	Tr1	<20	<40	5,210	11,620	1,370	220
JDV24	Tr1	<20	<40	12,540	14,370	420	230
JDV308	Tr1	70	<40	8,450	12,630	720	510
JDV94	Tr1	<20	<40	6,870	12,470	1,110	
JDV81	Th0	420	2,140	6,250	510	1,680	
JDV6	Th1	640	<40	90	720	6,480	
JDV110	Th1	420	<40	<40	640	4,290	
Cells preincubated without IL-10							
JDV305	Th1	140	<40	1,220	450	2,520	220
JDV5	Th1	850	<40	<40	1,120	8,450	
JDV40	Th1	480	<40	210	720	4,240	
JDV60	Th1	570	<40	330	440	3,590	
JDV101	Th1	440	<40	150	320	6,540	

To generate antigen-specific T-cell clones, CD4⁺ T cells from donor JDV cells were stimulated with irradiated purified allogeneic monocytes in the presence or absence of IL-10 (100 U ml⁻¹). After 10 days, CD4⁺ T cells were cloned. The different T-cell clones were expanded and selected for their specificity against the same allogeneic monocytes. To measure cytokine production, T cells were activated with crosslinked anti-CD3 (10 μ g ml⁻¹) and anti-CD28 (1 μ g ml⁻¹) mAbs, supernatants were collected after 48 h of culture or 72 h for TGF- β . Results are expressed in pg ml⁻¹ and represent pooled data from four independent experiments.

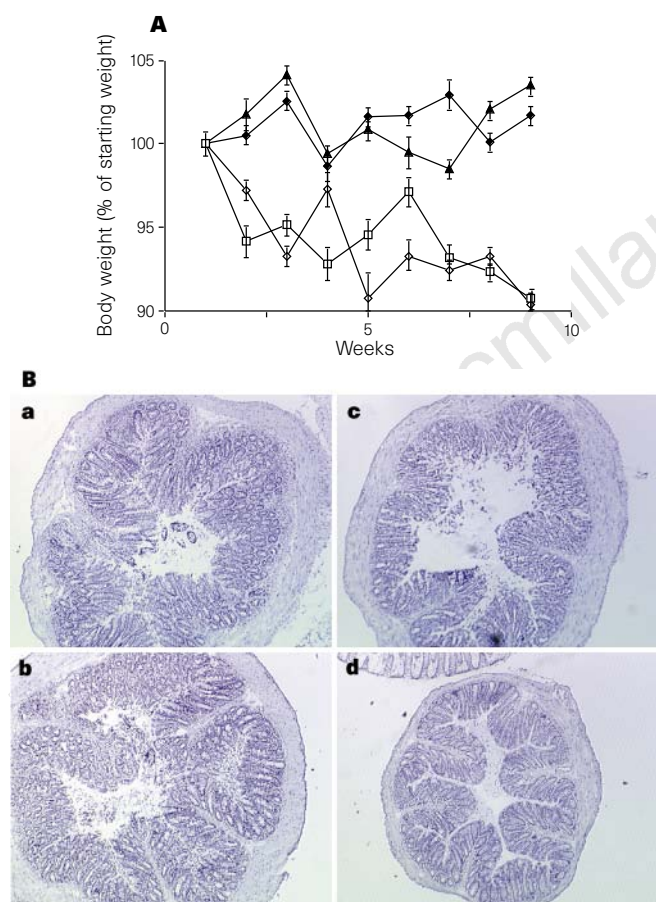


Figure 4 Co-transfer of Tr1 clones prevents colitis. **A**, Groups of 5 *scid* mice were reconstituted with 4×10^5 CD4⁺CD45RB^{hi} splenic T cells from 6-week-old BALB/c mice either alone (white squares) or in the presence of either 2×10^5 CD4⁺CD45RB^{lo} T cells (black triangles) or 2×10^5 OVA-specific Tr1 clones (A-10-9) (white diamonds for animal not fed with ovalbumin, and black diamonds for animals fed with ovalbumin). Half of the groups were fed with ovalbumin diluted in the drinking water (100 ng ml⁻¹). Feeding with ovalbumin did not change the pathogenic effect of CD4⁺CD45RB^{hi} T cells or the protective effect of CD4⁺CD45RB^{lo} T cells, and only animals fed with ovalbumin are shown for these two groups. Data represent mean plus s.e.m. of 5 animals per group. Results are representative of two experiments. **B**, Severe colitis in mice restored with CD4⁺CD45RB^{hi} T cells (**a**) and with CD4⁺CD45RB^{hi} T cells and a Tr1 clone (A-10-9) but not fed with ovalbumin (**b**). Protection from colitis in mice reconstituted with either CD4⁺CD45RB^{hi} T cells plus CD4⁺CD45RB^{lo} T cells (**c**) or in mice reconstituted with CD4⁺CD45RB^{hi} T cells and a Tr1 clone and fed with ovalbumin (**d**).

the presence of IL-4 had no suppressive effects, but rather enhanced OVA-induced proliferation of naive CD4⁺ T cells.

Addition of neutralizing anti-IL-10 and anti-TGF- β antibodies augmented the proliferative responses of naive human and mouse CD4⁺ T cells in the presence of Tr1 cells (Fig. 3). These data demonstrate that IL-10 induces the *in vitro* differentiation of a new regulatory CD4⁺ T-cell subset, which suppresses antigen-specific T-cell responses in both mice and man. These suppressive activities are predominantly mediated by IL-10 and TGF- β . Moreover, similar to the data previously reported for T cells stimulated in the presence of exogenous IL-10 (ref. 12), naive T cells stimulated in the presence of Tr1 clones fail to proliferate and to secrete IL-2, IL-4, IL-5 and IFN- γ after re-stimulation (not shown).

We sought to establish whether these Tr1 clones were functional *in vivo* and could regulate a pathogenic T-cell response. IL-10 is important for the maintenance of T-cell tolerance in the gut, as IL-10-deficient mice develop inflammatory bowel disease, which is thought to be mediated by normal enteric antigens¹³. In addition, administration of IL-10 to young IL-10-deficient mice prevents inflammatory bowel disease¹⁴. IL-10 also significantly inhibits the development of inflammatory bowel disease (IBD) induced by the transfer of CD4⁺CD45RB^{hi} D4⁺ T cells into SCID mice¹⁵. Here we show, in this latter model of IBD induction, that transfer in SCID mice of as few as 2×10^5 OVA-specific Tr1 cells (A-10-9 (Fig. 4) or A-10-11 (data not shown)) prevents IBD induced by pathogenic CD4⁺CD45RB^{hi} splenic T cells. Not surprisingly, the Tr1 cells are effective only upon stimulation *in vivo* by feeding the mice with OVA (Fig. 4). No difference in the pathogenicity of the CD4⁺CD45RB^{hi} splenic T cells or in the protective effects of the CD4⁺CD45RB^{lo} T cells¹⁶ was observed after feeding the mice with OVA in the absence of Tr1 cells (Fig. 4). These results demonstrate that Tr1 clones can prevent a T-cell-mediated disease *in vivo*. Furthermore, they indicate that the Tr1 cells must be activated *in vivo* to be effective. Finally, because the function of Tr1 cells is mediated by soluble factors, the present findings suggest that they can suppress active immune responses to unknown antigens in the microenvironment by an antigen-driven bystander suppression mechanism.

Transfer of CD4⁺ T-cell clones isolated from the gut of SJL mice after oral tolerance induction with myeloid basic protein (MBP)¹⁷ or from the pancreas of NOD mice¹⁸ has been shown to suppress experimental autoimmune myelitis and diabetes, respectively. These inhibitory activities were relieved by anti-TGF- β , suggesting that these clones mediated their suppression through TGF- β ^{17,18}. Although these T-cell clones appear to have similar functions to Tr1 cells, their cytokine production profiles are different. The T-cell clones isolated from SJL and NOD mice produce low levels of IL-10 and high levels of biologically active TGF- β , whereas the T-cell clones isolated from MBP transgenic mice fed with MBP (called Th3

cells) exclusively produced active TGF- β and no IL-10, IL-4, IL-2 or TNF- α ¹⁹. In contrast, Tr1 cells consistently produce low levels of active TGF- β and high levels of IL-10. Another difference is that both cytokines contribute to the suppressive activity of Tr1 cells. Taken together, these data suggest that Th3 and Tr1 cells are distinct types of CD4⁺ regulatory T cells.

The isolation of Tr1 cells from the peripheral blood of SCID patients, in which high levels of IL-10 *in vivo* are associated with tolerance after allogeneic stem-cell transplantation¹⁰, and from anergic T cells stimulated by major histocompatibility complex (MHC) class II-positive melanoma cells²⁰, supports the hypothesis that Tr1 cells are generated *in vivo* by chronic exposure of naive T cells to alloantigens in the presence of IL-10. Furthermore, based on the *in vitro* immunosuppressive activities of Tr1 cells, it is possible that these Tr1 cells specific for the host antigens have similar properties *in vivo*, and therefore may be responsible for transplantation tolerance in man. The observation that IL-10 production is associated with tolerance in murine models of organ and stem-cell transplantation is consistent with this notion^{21,22}.

However, administration of IL-10 failed to induce T-cell tolerance in other experimental models of transplantation^{23–26} and autoimmune disease, such as experimental autoimmune encephalomyelitis²⁷ or autoimmune haemolytic anaemia²⁸, which could be explained by our observations suggesting that the potential induction of tolerance through differentiation of Tr1 cells is a lengthy process *in vivo*, requiring chronic antigen-specific stimulation in the presence of IL-10. Thus systemic administration of IL-10 after the onset of the disease may not impede the effector phase of the immune response, and may therefore not result in tolerance.

Our findings indicate that stimulation of CD4⁺ T cells in the presence of IL-10 generates a subset of CD4⁺ T cells that inhibits antigen-specific immune responses through the secretion of IL-10 and TGF- β . The cytokine profile appears to be stable, as Tr1 clones cultured for more than 12 months produced the same cytokine profile after activation. All regulatory cells described previously have been isolated after *in vivo* priming with antigens. The ability to generate Tr1 cells after exposure to antigen *in vitro* may be an advantage for further functional analysis and potential clinical applications, particularly because we have demonstrated their regulatory capacity *in vivo*. Moreover, because Tr1 cells suppress immune responses directed against other antigens in the microenvironment, they could potentially be used to regulate T-cell-mediated disease, even when the pathogenic antigen is unknown. □

Methods

Cloning of Tr1 cells. Human Tr1 cells were generated from peripheral blood CD4⁺ T cells stimulated with allogeneic monocytes in the presence of exogenous IL-10. After 10 days, the CD4⁺ T cells were cloned by flow cytometry. Cells were labelled with an anti-CD4-FITC monoclonal antibody (Becton Dickinson, Mountain View, CA) and the viable cells were sorted at one cell per well in wells pre-coated with anti-CD3 monoclonal antibody (SPV-T3 (ref. 12) 100 μ g ml⁻¹ in 100 μ l Tris, 0.1 M, pH 9.4). After sorting, a mixture of irradiated feeder cells (JY, 10⁵ per ml and PBMC, 10⁶ per ml) and 10 U ml⁻¹ of rIL-2 in 100 μ l were added. Clones were expanded with IL-2 (10 U ml⁻¹) and by repetitive stimulation with high doses of crosslinked anti-CD3 monoclonal antibody (100 μ g ml⁻¹). The cloning of the mouse Tr1 cells was performed by limiting dilution at 0.3 cells per well in wells coated with 50 μ g ml⁻¹ of anti-CD3 monoclonal antibody (2C11, PharMingen, San Diego) in PBS and containing irradiated total splenocytes (10⁷ per ml) and 20 U ml⁻¹ IL-2. Clones were expanded by culture with IL-2 (20 U ml⁻¹) and IL-4 (20 U ml⁻¹), OVA peptide (2 μ M) and irradiated splenic APCs (10⁷ per ml).

Flow cytometry analysis of intracellular cytokines. Analysis of intracellular cytokines by flow cytometry was performed as described²⁹. Cells (10⁶ per ml) were activated with immobilized anti-CD3 and anti-CD28 monoclonal antibodies (PharMingen, San Diego) for 4 h. Brefeldin A (Sigma, St Louis) was added at 10 μ g ml⁻¹, and 2 h later cells were collected, washed and fixed with

formaldehyde (2%). For intracellular staining, cells were incubated with the following monoclonal antibodies (PharMingen, San Diego): anti-IL-4-FITC or PE (11B11, 5 μ g ml⁻¹), anti-IFN- γ -PE (XMG1.2, 5 μ g ml⁻¹), anti-IL-5-PE (TRFK5-2, 5 μ g ml⁻¹), anti-IL-10-FITC (JES-16E3, 5 μ g ml⁻¹), anti-IL-2-PE (JES6-5H4, 2.5 μ g ml⁻¹) or the anti-clonotype KJ1-26-FITC (5 μ g ml⁻¹). Samples were analysed on a FACScan (Becton Dickinson, Mountain View, CA).

Cytokine analysis. Human CD4⁺ T-cell clones or populations at 1 $\times$ 10⁶ per ml were stimulated by immobilized anti-CD3 (10 μ g ml⁻¹) and anti-CD28 monoclonal antibodies (1 μ g ml⁻¹) for 48 h. The production of IL-2, IL-4, IL-5, IL-10 and IFN- γ was measured by immunoenzymatic assays, as described¹². The amounts of IL-2, IL-4, IL-10 and IFN- γ produced by murine CD4⁺ T-cell clones was measured by ELISA in supernatants collected 48 h after culture of the T cells (10⁶ per ml) stimulated with OVA-peptide (1 μ M) and irradiated total splenic APCs, as described⁹. For TGF- β measurements, cells were cultured for 72 h in Yssel's medium³⁰ without serum and the amount of TGF- β was measured by ELISA (R and D System, Minneapolis, MN) after an acid activation, according to the manufacturer's instructions.

Proliferation assays. All proliferation assays were carried out in Yssel's medium³⁰ supplemented with 10% FCS and 1% human serum for human cells or with 10% FCS for murine cells. Alloantigen-specific stimulation was performed by culturing human CD4⁺ T-cell clones (10⁶ per ml) with purified irradiated (4,000 rad) monocytes (10⁶ per ml) in 200- μ l round-bottomed 96-well plates (Linbro, ICN Biomedicals, Aurora, OH). T-cell proliferation was measured after 5 days incubation at 37 °C and 5% CO₂ and a 12-h pulse with [³H]-1 μ R. Activation of human CD4⁺ T cells with immobilized anti-CD3 and anti-CD28 mAb was performed as described¹². In brief, antibodies were diluted at the indicated concentration in 0.1 M Tris buffer, pH 9.4, and plates incubated for a week at 4 °C. After washing the plates, CD4⁺ T cells were added at a concentration of 5 $\times$ 10⁴ cells per well and proliferation was measured after 3 days by [³H]-TdR uptake. Similarly, murine CD4⁺ T-cell populations or T-cell clones were stimulated at a concentration of 5 $\times$ 10⁴ cells per well with OVA peptide (0.5 μ M) and irradiated (4,000 rad) total splenocytes (5 $\times$ 10⁵ cells per well) for 3 days.

Inflammatory bowel disease. The induction of IBD was performed as described¹⁶. In brief, C.B-17 *scid* mice 8–12 weeks old. (Simonsen Laboratories, Gilroy, CA) were injected intraperitoneally with 100 μ l of PBS containing 4 $\times$ 10⁵ CD4⁺CD45RB^{hi} sorted splenic T cells with or without either 2 $\times$ 10⁵ CD4⁺CD45RB^{lo} splenic T cells or 2 $\times$ 10⁵ Tr1 clones. Half of the mice were fed with OVA diluted in the drinking water at 100 ng ml⁻¹. Weight gain or loss, as a percentage of original starting weight, was scored weekly for 9 weeks. For preparation of tissue for histopathologic analysis, approximately 10-mm segments of the distal large intestine were removed and fixed in a solution of 10% formalin. Fixed tissues were frozen and sections were prepared and stained with haematoxylin.

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Lipopolysaccharide-binding protein is required to combat a murine Gram-negative bacterial infection

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An invading pathogen must be held in check by the innate immune system until a specific immune response can be mounted. In the case of Gram-negative bacteria, the principal stimulator of the innate immune system is lipopolysaccharide (LPS), a component of the bacterial outer membrane¹. *In vitro*, LPS is bound by lipopolysaccharide-binding protein (LBP)² and transferred to

CD14—the LPS receptor on the macrophage surface^{3,4}—or to high-density lipoprotein (HDL) particles^{5,6}. Transfer to CD14 triggers an inflammatory response which is crucial for keeping an infection under control. Here we investigate how LBP functions *in vivo* by using LBP-deficient mice. Surprisingly, we find that LBP is not required *in vivo* for the clearance of LPS from the circulation, but is essential for the rapid induction of an inflammatory response by small amounts of LPS or Gram-negative bacteria and for survival of an intraperitoneal *Salmonella* infection.

The mouse LBP gene was disrupted in embryonic stem cells by homologous recombination. The mutant cells were injected into BALB/c blastocysts and a resulting mosaic male was selected to generate heterozygous and homozygous LBP-deficient animals (Fig. 1). Serum from animals heterozygous for the mutation contained LBP detectable by enzyme-linked immunosorbent assay (ELISA), but the serum from homozygous knockout mice did not (Table 1). LBP function was measured as the capacity to transfer LPS labelled with fluorescein isothiocyanate to mouse CD14 expressed on the surface of CHO cells; this was readily demonstrated in LBP^{+/+} and LBP^{+/-} sera but none is detectable in the serum of the LBP^{-/-} homozygotes (Fig. 2). By these criteria, homologous recombination has resulted in a complete loss of LBP activity in our homozygous mutant animals.

Both heterozygous and homozygous LBP-deficient animals appear normal and are fertile. A preliminary analysis of tissue from all main organs of male and female LBP^{-/-} mice at 14 weeks of age revealed no differences compared with an LBP^{+/+} age-matched littermate. LBP may be involved in lipid transfer reactions⁷, so we determined the concentrations of total serum cholesterol and triglycerides in heterozygous and homozygous animals: the

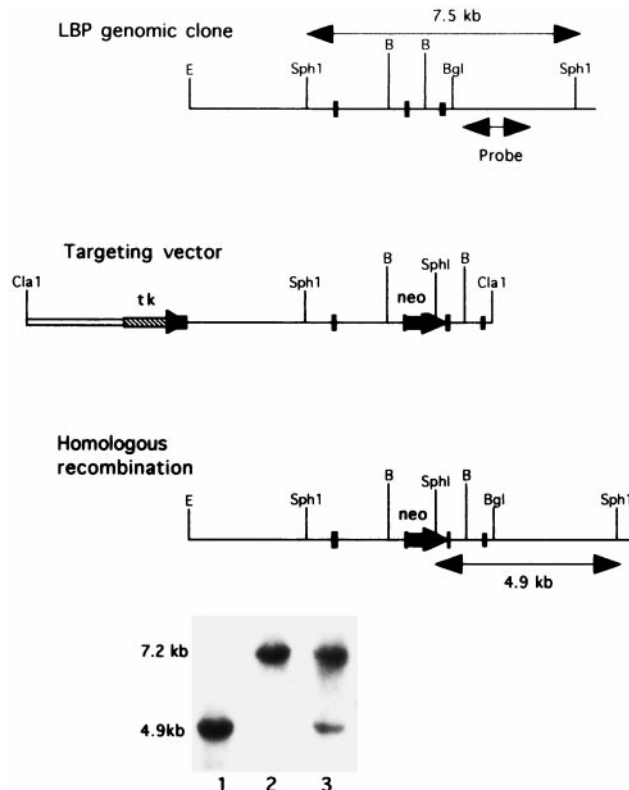


Figure 1 Maps of the mouse genomic clone containing three exons of the LBP gene (top), the targeting vector (middle) and the expected structure of the recombinant chromosome (lower). Southern analysis of three F₂ littermates: homozygous LBP-deficient animal (lane 1), wild-type (lane 2) and heterozygote (lane 3).

Table 1 LBP concentration in sera of F_2 animals

Genotype	LBP (ng ml ⁻¹)
LBP ^{+/+}	113.5 ± 10.7 (n = 8)
LBP ^{+/-}	94.9 ± 12.3 (n = 18)
LBP ^{-/-}	Not detectable (n = 7)

values from these two groups were indistinguishable (cholesterol: LBP^{+/+}, 89.3 ± 3.6 mg dl⁻¹; LBP^{-/-}, 92.1 ± 4.8 mg dl⁻¹; triglycerides: LBP^{+/+}, 127 ± 7 mg dl⁻¹; 113 ± 6 mg dl⁻¹). In addition, we analysed the distribution of serum lipoproteins by isotachopheresis⁸⁻¹⁰. There were no significant differences in the patterns of serum lipoproteins from LBP^{-/-} and LBP^{+/+} animals, even when the animals were fed on high-fat diets. Thus, we see no evidence for any essential non-redundant role for LBP in serum lipoprotein metabolism in mice.

It has been suggested that LBP transfers LPS to HDL⁶ and thus affects the clearance of LPS from the circulation. To determine whether LBP plays this role in the mouse, we injected 1 µg (0.9 MBq) ¹²⁵I-labelled LPS¹¹ intravenously into LBP^{+/+} and into LBP^{-/-} mice: its clearance was followed by whole-body scintigraphy. In both groups of mice, the LPS was cleared rapidly from the circulation and more than 95% of it was concentrated in the liver within 5 min. LBP is therefore not essential for the clearance of LPS from the circulation in mice.

We next investigated whether the rate of transfer *in vitro* of LPS by LBP to the monocyte/macrophage LPS receptor CD14 (ref. 2), which causes cellular activation and the release of proinflammatory mediators, is reflected in a requirement for LBP in the induction of an inflammatory response *in vivo*. This response is dependent on CD14 because it is abrogated in CD14-deficient animals¹². Furthermore, injection of large amounts of IgG prepared from a rabbit anti-mouse LBP serum blocks induction of the inflammatory response, suggesting that LBP is directly involved in this process¹³. Mice treated with galactosamine are extremely sensitive to the toxic effects of low doses of LPS¹⁴. Galactosamine-sensitized LBP^{+/+} animals injected intraperitoneally (i.p.) with 200 ng LPS showed symptoms of acute illness within 5 h and died 7–12 h after administration of LPS. In contrast, LBP^{-/-} animals showed no symptoms of shock and they survived (Fig. 3a). The serum concentration of tumour-necrosis factor-α (TNF-α) in LBP^{+/+} mice bled 90 min after i.p. injection of galactosamine and LPS was 1,110 ± 340 pg ml⁻¹

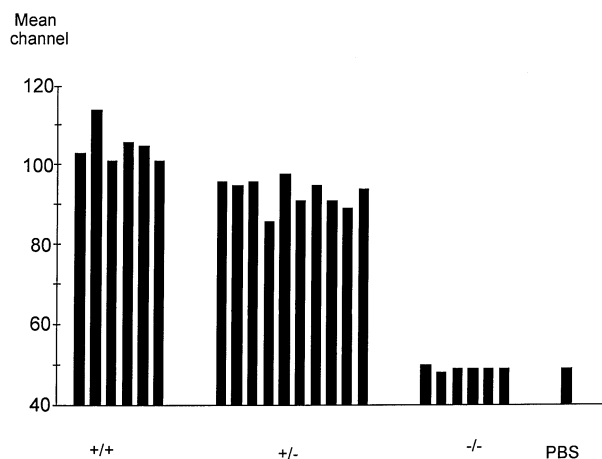


Figure 2 FACS analysis of LBP-mediated transfer of FITC-labelled LPS to CHO cells expressing mouse CD14. Sera from homozygous LBP^{-/-}, LBP^{+/+} and from LBP^{+/+} littermates were tested.

Table 2 Bacterial load in peritoneum, liver and spleen of LBP^{-/-} and LBP^{+/+} mice

	Day 2	Day 3
Peritoneum LBP ^{-/-}	49.7 ± 14.5 × 10 ³	2,126.8 ± 1,555.4 × 10 ³
Peritoneum LBP ^{+/+}	2.9 ± 0.5 × 10 ³	10.3 ± 5.7 × 10 ³
Liver LBP ^{-/-}	111.2 ± 30.9 × 10 ³	728.5 ± 402.0 × 10 ³
Liver LBP ^{+/+}	10.1 ± 4.0 × 10 ³	13.6 ± 4.7 × 10 ³
Spleen LBP ^{-/-}	71.3 ± 20.2 × 10 ³	894.0 ± 85.7 × 10 ³
Spleen LBP ^{+/+}	5.5 ± 2.5 × 10 ³	8.0 ± 2.5 × 10 ³

Bacteria were counted two and three days after i.p. infection with 1–3 × 10² CFU *S. typhimurium*. Five animals per group. Values shown are mean ± s.e.m.

(n = 4), whereas LBP^{-/-} animals had less than 10 pg TNF-α per ml (n = 4), indicating that the latter's capacity to mount an inflammatory response when challenged with LPS is severely impaired.

LBP mediates a response not only to LPS, but also to intact Gram-negative bacteria^{15,16}. Our LBP-deficient mouse can therefore be used to explore the role of an LBP-mediated inflammatory response in host defence against a Gram-negative bacterial infection. We injected a low dose (1–3 × 10² CFU) of *Salmonella typhimurium* i.p. into LBP^{+/+} and into LBP^{-/-} animals and tested these animals for their ability to control the spread of the injected bacteria. After 48 h, groups of five animals were subjected to peritoneal wash and the number of bacteria present determined by plating; the numbers of bacteria in liver and spleen were measured at the same time. The procedure was repeated with animals injected for 72 h. By 48 h, we found that the peritoneal wash of LBP-deficient animals contained approximately tenfold more bacteria than controls, as did the liver and spleen (Table 2).

The inability of the LBP^{-/-} animals to check the multiplication of the bacteria over the initial period has dramatic consequences for the course of the infection. LBP^{-/-} animals injected i.p. with 1–3 × 10² CFU of *S. typhimurium* died within the first 5 days, whereas 10 out of 12 heterozygous controls survived for longer than three weeks (Fig. 3b). The surviving control animals showed no signs of illness, although moderate loads of *Salmonella* were present both in liver and spleen.

The cause of death of LBP-deficient animals in this *Salmonella* infection model is unclear, but in our experience animals with a bacterial load in the liver of ≥10⁹ CFU die within 24 h. Tissue sections from the principal organs of LBP^{-/-} animals examined over the course of the infection showed progressive inflammatory foci, particularly in the liver.

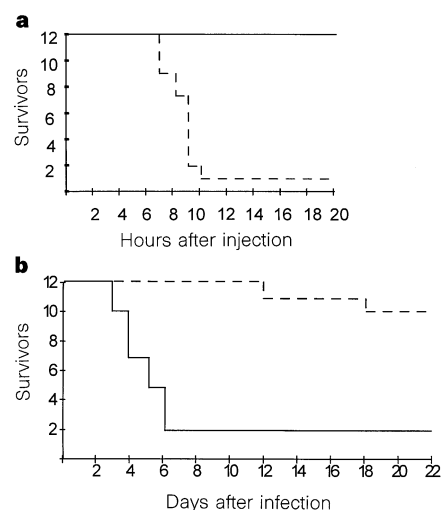


Figure 3 Survival of LBP^{-/-} (solid line) and of LBP^{+/+} (broken line) animals challenged **a**, with 50 mg galactosamine and 200 ng of LPS i.p. and **b**, with 100–300 CFU *S. typhimurium*.

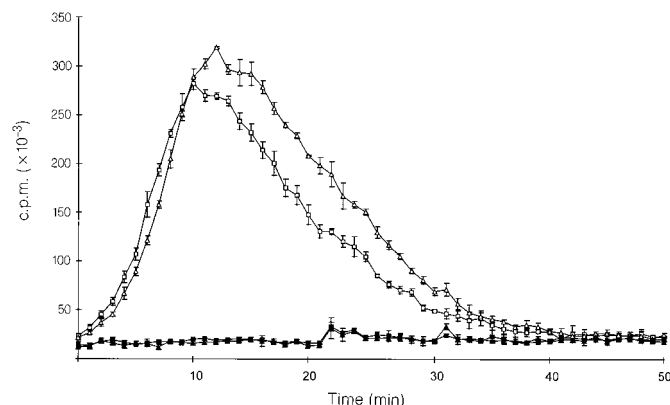


Figure 4 Oxidative-burst response of peritoneal wash cells from LBP^{+/-} (□, ■) and LBP^{-/-} (Δ, ▲) mice challenged *ex vivo* with *S. typhimurium* in the presence of serum from LBP^{+/-} (□, Δ) or from LBP^{-/-} (■, ▲) animals.

Table 3 Cellular composition of peritoneal wash during infection

Mouse	Hours after infection	Granulocytes	Macrophages	Lymphocytes
LBP ^{+/-}	24	37 ± 3	55 ± 4	7 ± 1
LBP ^{+/-}	24	39 ± 5	52 ± 4	9 ± 3
LBP ^{+/-}	24	29 ± 3	56 ± 6	16 ± 3
LBP ^{-/-}	48	54 ± 2	37 ± 5	9 ± 3
LBP ^{-/-}	48	64 ± 2	31 ± 3	6 ± 1
LBP ^{+/-}	48	40 ± 6	45 ± 2	16 ± 2
LBP ^{+/-}	48	46 ± 2	44 ± 4	10 ± 2

Values given are the percentage of total cells; $1-2 \times 10^6$ cells were recovered from each animal.

The first line of defence against a bacterial infection involves the initiation of an inflammatory response and the release of mediators such as oxygen radicals and proinflammatory cytokines from activated macrophages and granulocytes. A transitory rise in the serum concentration of TNF- α is a frequently used marker of inflammation induced by a bolus injection of LPS. Using a sensitive bioassay¹⁷, we detected no significant amount of TNF- α either in the serum or in the peritoneal wash of infected LBP^{+/-} and LBP^{-/-} animals. We therefore compared the ability of peritoneal cells from LBP^{-/-} and LBP^{+/-} mice to respond to *S. typhimurium*. The peritoneal washes from infected mice from both groups were similar in cellular composition (Table 3), but there was a clear difference when these were assayed for generation of an oxygen radical burst on exposure to *S. typhimurium ex vivo* (Fig. 4). Cells from LBP^{-/-} animals failed to respond to the bacterium when assayed in the presence of homologous serum but responded as well as cells from LBP^{+/-} mice in serum from LBP^{+/-} animals. Thus, LBP^{-/-} peritoneal-wash cells retain the intrinsic capacity to respond to the bacterium but fail to do so because of a lack of LBP in the extracellular fluid.

Our results indicate that LBP is required to induce a rapid inflammatory response to *S. typhimurium* in murine peritoneal cells and is essential for the survival of an intraperitoneally induced *S. typhimurium* infection. □

Methods

Generation of LBP-deficient mice. The LBP exon encoding alanine 43 to serine 55 was recovered in a genomic clone and disrupted by insertion of the bacterial neomycin-resistance gene. The targeting vector was transfected into the embryonic stem cell line 14.1. A homologous recombinant line was injected into BALB/c blastocysts which were transferred into pseudopregnant (CBAx57BL6)F1 females. One mosaic male was crossed to BALB/c and F₁ progeny were screened by Southern blotting. Those carrying the disrupted gene were crossed *inter se* to generate homozygous LBP-deficient animals.

LBP assays. For ELISA, recombinant histidine-tagged mouse LBP was prepared as described¹⁵ and used to raise a polyclonal antiserum in rabbits. IgG was purified from the serum and used to coat ELISA plates, and was biotinylated for use as the detecting antibody. The detection limit was 5 ng LBP ml⁻¹.

Transfer of fluorescein isothiocyanate (FITC)-LPS to transfected CHO cells expressing mouse CD14 on the surface was determined as described¹⁶.

Serum cholesterol, triglyceride and lipoprotein analysis. LBP^{+/-} and LBP^{-/-} mice fasted overnight and were then bled. Total serum cholesterol ($n = 20$ per group) and total serum triglycerides ($n = 14$ per group) were determined using commercially available kits (Sigma). For lipoprotein analysis, groups of LBP^{+/-} and LBP^{-/-} mice ($n = 8$ per group) were fed on one of three diets (4% fat; 10% fat, 0.014% cholesterol; 21% fat, 0.15% cholesterol) for 7 days. Food was then removed for 12 h and animals were bled. Sera were analysed by isotachopheresis as described^{8,9,10}; this system allows five lipoprotein subclasses to be discriminated in the mouse. Individual peak areas were calculated and expressed as a percentage of the total signal; ratios of the signal in α -HDL to that in pre- β -HDL were used for intergroup comparison.

Challenge with LPS and bacteria. Mice 10–12 weeks old were sensitized to LPS by i.p. injection of 50 mg galactosamine. They were given 200 ng LPS (*Salmonella abortus equi*) simultaneously. Injected animals died within 12 h. Animals receiving either galactosamine alone ($n = 3$) or LPS ($n = 3$) were unaffected.

S. typhimurium was grown in L broth to mid-log phase and counted by plating. Mice were infected i.p. with $1-3 \times 10^2$ CFU. Survival was followed for a period of three weeks.

Analysis of peritoneal wash cells. Cells were washed from the peritoneal cavity in 10 ml ice-cold RPMI 1640 medium, washed once and resuspended in the same medium to a density of 1×10^7 ml⁻¹.

Cell composition. Peritoneal wash cells were prepared from two infected LBP^{+/-} at 24 h and 48 h after i.p. infection with $1-2 \times 10^2$ *S. typhimurium*. Peritoneal cells were similarly prepared from one LBP^{-/-} animal 24 h after infection and from two LBP^{-/-} animals 48 h after infection. The cell composition was determined by haematoxylin–eosin staining: 300 cells per slide and four slides per animal were counted; results of the four slides were averaged (values are mean $\pm$ s.d.).

Oxidative-burst response. The oxidative-burst response was measured as described¹⁸. Briefly, 8×10^6 peritoneal-wash cells were equilibrated at 37 °C for 30 min in RPMI 1640 with 1×10^{-4} M luminol in the presence of 10% serum from LBP^{+/-} or LBP^{-/-} mice. 1×10^8 CFU *S. typhimurium* were then added and chemiluminescence measured in a Berthold LB953 luminometer.

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Imprinted expression of the *Igf2r* gene depends on an intronic CpG island

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Gametic imprinting is a developmental process that induces parental-specific expression or repression of autosomal and X-chromosome-linked genes^{1,2}. The mouse *Igf2r* gene (encoding the receptor for insulin-like growth factor type-2) is imprinted and is expressed from the maternal allele after embryonic implantation³. We previously proposed that methylation of region 2, a region rich in cytosine-guanine doublets (a 'CpG island') in the second intron of *Igf2r*, is the imprinting signal that maintains expression

of the maternal allele⁴. Here we use mouse transgenes to test the role of region 2 and the influence of chromosome location on *Igf2r* imprinting. Yeast artificial chromosome transgenes successfully reproduced the imprinted methylation and expression pattern of the endogenous *Igf2r* gene; deletion of region 2 from these transgenes caused a loss of imprinting and restored biallelic *Igf2r* expression. These results define a primary role for region 2 and a negligible role for chromosomal location in *Igf2r* imprinting; they also show that methylation imprints can maintain allelic expression. Short transgenes containing only region 2 and yeast artificial chromosome transgenes with an inactive *Igf2r* promoter do not attract parental-specific methylation. All transgenes showing paternal-specific repression of *Igf2r* produced an antisense RNA whose transcription was dependent on region 2. The production of an antisense RNA by the repressed parental allele is reminiscent of the imprinting of the *Igf2/H19* gene pair⁵ and may indicate that expression competition could play a general role in imprinting.

The current model of the imprinting mechanism suggests that an imprinting signal, inherited during male or female gametogenesis, acts on one gene or a cluster of genes to generate allele-specific expression in diploid cells. The demonstration that transgenes of the imprinted *H19* gene^{6,7} can maintain imprinted expression at other chromosomal locations suggests that imprinting signals are closely linked to the affected genes. However, it is also clear from deletions of the mouse *H19* gene and deletions in the human Prader-Willi/Angelman syndromes^{8–10} that long-range *cis* effects are active in imprinting. A role for long-range effects and/or the chromosome location was also indicated by the clustering of imprinted genes into regions that show parental-specific differences in replication timing and recombination frequency^{11,12}.

Although, a common imprint sequence motif has not been identified among the twenty-two genes known to be imprinted^{1,2}, we have proposed that C + G-rich sequences that resemble CpG islands¹³ and which are associated with many imprinted genes and are often subject to parental-specific methylation, could act as a common imprinting element without a requirement for direct

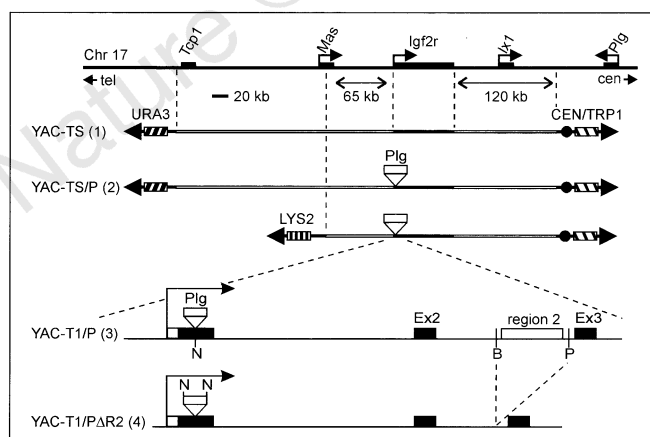


Figure 1 Cloning of YACs in *S. cerevisiae*. Top line shows the physical map (black boxes indicate genes) around the *Igf2r* locus²⁵ on mouse chromosome 17 and alignment of the YAC constructs YAC-TS, YAC-TS/P, YAC-T1/P and YAC-T1/PΔR2. YAC-TS (step 1) was modified by inserting a fragment of mouse Plasminogen cDNA (Plg) into the *NotI* site (N) in exon 1 of the *Igf2r* gene to give YAC-TS/P (step 2). This was truncated at the *Mas* locus to derive the 300-kb YAC-T1/P. YAC-T1/P was modified by deleting region 2 (steps 3 and 4), which gave YAC-T1/PΔR2. Correctly modified YAC clones were identified by standard Southern blot analysis and by PFGE. *SalI* fragments from 20–80 kb were unchanged in the YACs, and *EcoRI*, *HindIII*, *PstI* fragments (0.5–6.5 kb) were correctly modified around the *Igf2r* promoter and region 2 (data not shown).

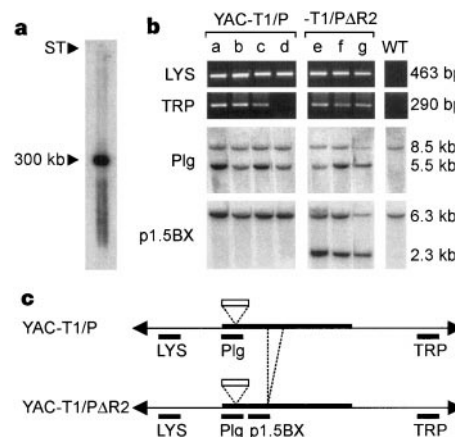


Figure 2 a, YAC DNA for microinjection (20 μl) separated by PFGE was hybridized with Plg cDNA; the 300-kb signal indicates intact YAC molecules (ST; slot). b, Analysis of transgene tail DNA of region-2-positive YAC-T1/P lines (a, b, c and d) and region-2-deleted YAC-T1/PΔR2 lines (e, f and g) by PCR of the YAC arms (LYS, TRP) and by blot-hybridization for *Igf2r* exon 1 (Plg) and region 2 (p1.5BX) to demonstrate the integrity of the YAC transgenes after integration into the mouse genome (WT, wild type). Line d lacked the TRP1 arm. The presence of the Plg-modified *Igf2r* exon 1 is shown by the 5.5-kb transgene-specific fragment; the 8.5-kb band is the endogenous Plasminogen gene. The 2.3-kb fragment identified by p1.5BX results from the deletion of region 2 from YAC-T1/PΔR2 transgenes (the endogenous region-2 fragment is 6.3 kb). c, The location of probes relative to the 300-kb YAC-T1/P and the 296-kb YAC-T1/PΔR2.

sequence homology¹⁴. The mouse *Igf2r* gene (for the insulin-like growth factor type-2 receptor, also known as the cation-independent mannose-6-phosphate receptor) contains in the second intron a 2-kilobase (kb) CpG island known as region 2, proposed to be an imprinting element⁴. It inherits a methylation mark from the female gamete which remains restricted to the maternal chromosome in diploid cells in the embryonic and adult stages. The remainder of a 130-kb region containing the 93-kb-long *Igf2r* gene is either equally methylated or, acquires allelic methylation late in development⁴. All imprinted genes examined so far are associated with patches of allele-specific methylation in adult tissues, but only three genes, *Igf2r*, *H19* and *Xist*, have been linked to germline-specific methylation imprints¹⁵. The significance of methylation in the imprinting mechanism has been demonstrated by the loss of allele-specific expression of *Igf2r*, *Igf2*, *H19* and *Xist* in mice deficient in genomic methylation following targeted mutation of the DNA methyltransferase gene^{16,17}.

The 450-kb yeast artificial chromosome construct YAC-TS, spanning the complete 93-kb *Igf2r* locus, was isolated from the ICRF C57BL6 YAC library¹⁸ using a Tcp1 (T complex protein 1, ref. 25) complementary DNA. YAC-TS was tagged (to disrupt the reading frame and provide a unique marker) by insertion of a Plg (plasminogen²⁵) fragment into the first exon of *Igf2r*. The YAC was shortened to 300 kb and shown to be collinear with the genomic map (data not shown) and renamed YAC-T1/P (Fig. 1). Four independent YAC-T1/P founder mice, lines a, b, c and d (Fig. 2b, c) were analysed for YAC-linked markers, bred with wild-type

C57BL6/CBAF1 mice, and expression of the YAC-T1/P-*Igf2r* allele was analysed after transmission through the male and female germ line. Imprinted *Igf2r* expression was studied in offspring hemizygous for the transgene in all major organs of adult mice and in day-13.5 embryos, by northern blot analysis (Fig. 3a) and RNase protection assay (Fig. 3b, c) using double-stranded and strand-specific probes based on the Plg tag inserted into the YAC-*Igf2r* allele. Northern analysis using a double-stranded Plg probe identified a 9-kb YAC-specific RNA in heart tissue following maternal transmission (heart is the principal site of *Igf2r* expression in adult mice³), and a marginally larger RNA following paternal transmission of the transgene (Fig. 3a), initially suggesting a lack of imprinting. However, analysis of the same RNA samples using the strand-specific riboprobe 1 (which specifically detects YAC-*Igf2r* messenger RNA) identified an RNA whose expression was seen only on maternal transmission (Fig. 3b, lanes M). Riboprobe 1 did not detect any mRNA from paternally inherited YAC transgenes (Fig. 3b, lanes P). The paternally expressed YAC-specific RNA (Fig. 3a, Plg) was shown, by using the strand-specific riboprobe 2, to be a transcript in reverse orientation to the *Igf2r* mRNA (referred to as an antisense RNA, or AS RNA). Riboprobe 2 identified the antisense RNA only in offspring with a paternally inherited YAC transgene (Fig. 3c, lanes P). All offspring with a maternally inherited YAC transgene lacked the antisense RNA (Fig. 3c, lanes M). The same antisense RNA is also expressed from the endogenous *Igf2r* locus at the exon 1/intron 1 boundary (Fig. 3c, lane 1)

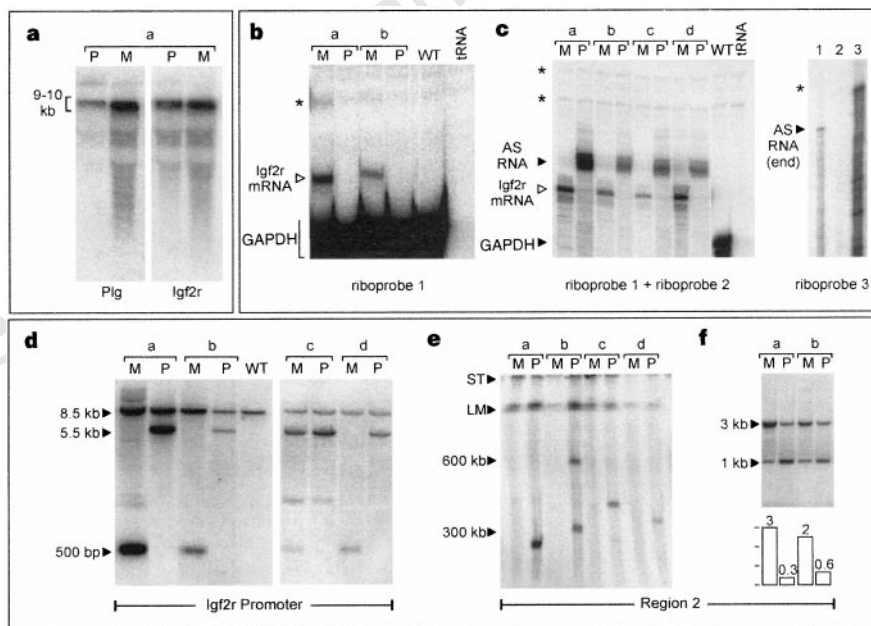


Figure 3 Parental-specific expression and methylation of YAC-T1/P transgenes. Four independent YAC-T1/P lines (a, b, c, d) were analysed after maternal (lane M) and paternal (lane P) transmission of the transgene. **a**, Heart RNA from line a, analysed by northern blot using a double-stranded Plg fragment (left) that identifies a transgene-specific signal, and the same filter rehybridized with a double-stranded probe spanning nt 500 to 1,500 of the 9-kb *Igf2r* cDNA (right) which identifies the endogenous plus transgene *Igf2r* signal. **b**, Heart RNA from line a and b transgenes, analysed by RNase protection and demonstrating maternal-specific expression of the YAC-*Igf2r* allele in adult heart. The probe, riboprobe 1, is specific for YAC-*Igf2r* mRNA and protected the expected 135-bp fragment following maternal transmission only. A GAPDH (glyceraldehyde-3-phosphate dehydrogenase) riboprobe (nt 794–698) was used as a loading control; an asterisk indicates the size of the input probe. **c**, RNase protection assay, demonstrating maternal YAC-*Igf2r* mRNA expression and paternal YAC-AS RNA expression in all four YAC-T1/P transgenes. YAC-*Igf2r* mRNA was assayed with riboprobe 1 and YAC-AS RNA with riboprobe 2: both are transgene-specific.

Lanes 1–3, RNase protection in wild-type RNA using riboprobe 3 (specific for the wild-type allele); lane 1, embryonic day 14 (end, endogenous AS RNA); lane 2, tRNA; lane 3, input riboprobe 3. **d**, Methylation of the YAC-*Igf2r* promoter. *Eco*RI- and *Not*I-digested DNA was hybridized with the Plg probe: a 5.5-kb fragment results if both *Not*I sites flanking the Plasminogen insert are methylated; the 0.5-kb band results if both these sites are unmethylated; the 8.5-kb fragment is the endogenous Plasminogen gene. **e**, Methylation of YAC region 2 assessed by *Mlu*I digestion, PFGE, and hybridization with the Plg probe. A single *Mlu*I site⁴ contained within region 2 is cut exclusively on paternal transmission of the transgene, as indicated by fragments (300–600 kb) entering the gel (LM, limiting mobility; ST, slots). **f**, DNA was digested with *Pvu*II and *Hpa*II and hybridized with a 1-kb *Mul*I-*Pvu*II fragment from region 2. The 3-kb band corresponds to methylated region 2, and the 1-kb band to unmethylated region 2. Signals from the endogenous and transgene loci are superimposed. The histogram below shows a quantification of the methylated (3 kb) to unmethylated (1 kb) signal using ImageQuant Software (Molecular Dynamics).

Table 1 Expression of YAC transgenes after maternal and paternal transmission

Transgene type	Transgene name	Copy number	Expression after maternal transmission	No. of offspring examined	Expression after paternal transmission	No. of offspring examined
YAC-T1/P	a	4–6	Igf2r mRNA	6	AS RNA	10
	b	2	Igf2r mRNA	19	AS RNA	11
	c	4	Igf2r mRNA [or AS RNA]	11 [12]	AS RNA	16
	d	2	Igf2r mRNA	2	AS RNA	6
YAC-T1/PΔR2	e	2	Igf2r mRNA	11	Igf2r mRNA	18
	f	4	Igf2r mRNA	3	Igf2r mRNA	3
	g	20	Igf2r mRNA	3	Igf2r mRNA	5

Expression of four YAC-T1/P and three YAC-T1/PΔR2 transgenic lines was examined following maternal or paternal transmission of the transgene. Imprinted expression of the YAC-Igf2r allele was maintained only in transgenes containing region 2 (YAC-T1/P lines a, b and d; the anomalous behaviour of line c is described in the text). YAC-T1/PΔR2 transgenes deleted for region 2 (lines e, f and g) expressed *Igf2r* mRNA biallelically. The copy number was estimated by the ratio of the PhosphorImager signal intensities of the Plg-tagged *Igf2r* exon 1 on the transgene to that of the endogenous Plasminogen locus.

specifically from the paternal chromosome (our unpublished data) and its involvement in repressing the paternal *Igf2r* allele is being assessed.

All the YAC transgenes analysed showed the expected³ tissue-specific expression pattern for *Igf2r* (data not shown). Three independent transgenes, lines a, b and d, showed the imprinted pattern of exclusive maternal-specific expression of *Igf2r* mRNA and exclusive paternal-specific expression of antisense RNA (Table 1) in embryonic and adult tissues. Furthermore, pedigrees from these three lines showed that imprinting was consistently reversed after passage through the germ line of the opposite sex in successive generations (data not shown). Lines a and b contained intact copies of YAC-T1/P, whereas line d lost the TRP1 arm but retained imprinted expression of *Igf2r* (Figs 2 and 3b, c). Males from line c did not pass the transgene to sons ($n = 56$), suggesting that it has been integrated into the X chromosome, but imprinting was preserved after parental transmission to daughters ($n = 26$), as shown in Fig. 3c (lane P). Maternal line c transmission ($n = 36$) resulted in male and female offspring which expressed only *Igf2r* mRNA (Fig. 3c, lane M), but also gave offspring expressing either the antisense RNA or both antisense RNA and *Igf2r* mRNA (data not shown), suggesting that there is a position effect at this integration site.

The 300-kb YAC-T1/P was modified to delete a 4-kb fragment containing all the CpG-rich sequences from region 2, and renamed YAC-T1/PΔR2. Region 2 is methylated at 28 CpGs spanning a 2-kb region⁴. Three transgenic YAC-T1/PΔR2 lines (e, f and g) containing intact copies were obtained (Table 1, and Fig. 2b). All three lines expressed Plg-tagged *Igf2r* mRNA following both maternal and paternal inheritance (Fig. 4a, b). Expression of *Igf2r* mRNA was comparable following maternal or paternal inheritance within any one line, and similar to that of *Igf2r* mRNA expressed from the YAC-T1/P transgenes that contained region 2. But, in contrast to the mice containing the region 2-positive YAC-T1/P transgene (Fig. 3), no antisense RNA was observed following paternal inheritance of the region-2-deleted YAC-T1/PΔR2 transgenes (Fig. 4a, b). The finding that deletion of region 2 restored paternal *Igf2r* mRNA expression but did not change maternal expression shows that region 2 is an imprinting element that appears to act only as a *cis*-repressor of the *Igf2r* promoter. Furthermore, whereas region 2 is not necessary for *Igf2r* mRNA expression, the lack of antisense RNA from the YAC-T1/PΔR2 transgenes indicates that region 2 is required for expression of this transcript and that a promoter and/or enhancer is contained in the 4-kb deletion. The negative correlation between antisense RNA production and region-2 methylation indicates that methylation has an inhibitory effect on this element.

To determine which part of the 4-kb element containing region 2 stimulates maternal-specific methylation, we generated a series of short transgenes centred around region 2 containing 1 kb (RII transgenes, five founder mice), 3 kb (P4 transgenes, five founders) or 14 kb of DNA (H/X-14 transgenes, two founders) (Fig. 5a). Methylation status was analysed following maternal and paternal

inheritance for three lines (two lines for H/X-14) for successive generations. Figure 5b–d shows that whereas all the region-2 short transgenes were methylated to various degrees, none showed any parental differences. The 1-kb RII transgene which contained the central repeat-rich part of region 2, previously proposed to be involved in attracting the gametic methylation imprint¹⁴, displayed a random methylation pattern in each litter (Fig. 5b). Offspring did not inherit the methylation status of the parent, instead hypomethylated and hypermethylated transgenes were found in each litter. Similarly, the P4 transgene was transmitted in a hypo- and hypermethylated form by both parents (Fig. 5c). However, P4 transgenes differed because their methylated status was stably inherited by the next generation. The 14-kb H/X-14 transgenes were relatively undermethylated, and this status was stably inherited in successive generations. The endogenous region 2 prevented analysis of these transgenes in oocytes or preimplantation embryos using a polymerase chain reaction (PCR) strategy¹⁹. However, the high-copy-number line-2 H/X-14 transgene (60–80 copies)

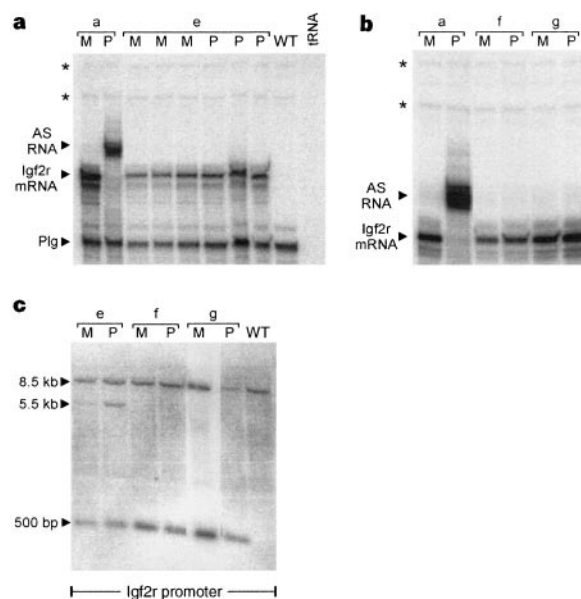


Figure 4 a and b, expression of the region-2-deleted YAC-T1/PΔR2 transgenes. Three independent lines (e, f, and g) analysed after maternal (lanes M) and paternal (lanes P) transmission of the transgene. **a, b**, Biallelic, non-imprinted expression of YAC-Igf2r mRNA in lines e, f and g transgenes in embryonic-day(E) 13.5 embryos using riboprobe 1 and riboprobe 2. Fetal liver endogenous Plg is used as a loading control. The imprinted YAC-T1/P line a was included as a control for riboprobe 2; asterisk indicates input probe. **c**, Methylation of the *Igf2r* promoter on the YAC-T1/PΔR2 transgene. Adult spleen DNA of transgenic mice was analysed as for Fig. 3d. A 0.5-kb fragment is seen after maternal and paternal inheritance in all three lines, indicating a lack of methylation regardless of the parental origin of the transgene. Line e is an exception (see text).

was analysed by DNA-blot hybridization and showed a low level of methylation at the *MluI* and *SfuI* sites in blastocysts after maternal inheritance (Fig. 5e).

Thus, short sequences around region 2 are insufficient to recapitulate the allelic methylation of the endogenous locus. These results contrast with those from CpG islands from non-imprinted genes which preserve their hypomethylated status as transgenes independent of transcription^{20,21}. Maintenance of methylation on region 2 may require other elements on the 300-kb YAC and expression from the *Igf2r* promoter may play a part. In support of this, two independent YAC transgenes inadvertently derived with an inactive *Igf2r* promoter (these transgenes failed to express *Igf2r* mRNA after insertion of the P_{lg} tag in the opposite transcription orientation to *Igf2r*; data not shown) were

not methylated at region 2 after maternal or paternal inheritance and, in keeping with the results shown in Fig. 3, expressed the antisense RNA following maternal and paternal inheritance.

Maternally inherited YAC-T1/P transgenes (lines a, b and d) expressed *Igf2r* mRNA from an unmethylated promoter but were methylated at region 2 and did not express antisense RNA. Paternally inherited transgenes showed the opposite pattern: expression of antisense RNA and lack of methylation at region 2, plus methylation of the *Igf2r* promoter and absence of *Igf2r* mRNA (Fig. 3d–f). This transgenic methylation/expression pattern mimics the behaviour of the endogenous locus, where methylation of region 2 is acquired in the female germ line but *Igf2r* promoter methylation is acquired later in embryonic development⁴. Most transgenes behaved like the endogenous locus, but offspring from YAC-T1/P line c and YAC-T1/PΔR2 line e expressed *Igf2r* mRNA after maternal transmission (Figs 3d, lane c(M) and 4c, lane e(M)), despite sparse methylation at the *Igf2r* promoter. We suggest that this reflects stochastic methylation in a minority of cells and that other factors such as chromosomal position can influence methylation of the *Igf2r* promoter, despite the fact that the YAC transgenes carry 65 kb of 5'-flanking DNA.

The mouse *Igf2r* gene lies in a 2.0-megabase region on chromosome 17 which shows a parental bias in replication timing²². As *Igf2r* can retain imprinted expression at different autosomal locations, these long-range effects are not dominant, and/or they may arise from the imprinted gene itself. The imprinted *H19* gene lies in a domain on chromosome 7 that also shows a replication-timing bias, and *H19* also retains imprinted behaviour at other chromosomal sites^{6,7}. Analysis of *Igf2r* and *H19* highlights the importance of short-range elements in the imprinting process, which is striking in view of the known clustering of imprinted genes which implicates long-range control elements. The *Igf2r* maternal germline methylation imprint on region 2 correlates with maternal repression of the antisense RNA and maternal expression of *Igf2r* mRNA. In contrast, the *H19* paternal germline methylation imprint correlates with paternal repression of the *H19* allele and with paternal expression of the flanking *Igf2* and insulin genes^{6–8}. Despite these differences, imprinting of the *Igf2r*/AS-RNA gene pair may be similar to that of *Igf2* and *H19*. Our result showing that repression of the *Igf2r* promoter can be relieved by deletion of region 2 has parallels with the de-repression of *Igf2* and insulin following deletion of the *H19* gene⁸. These results from two distinct imprinted loci support the concept of expression competition⁵ as a unifying mechanism in imprinting and indicate that the view of imprinting as one active and one silent parental allele may need revision. □

Methods

YAC modifications. A plasminogen cDNA fragment (nucleotides 2,105–2,572) was inserted into the *NotI* site in *Igf2r* exon 1 by homologous transformation of YAC-TS in *Saccharomyces cerevisiae* AB1380. A fragmentation vector²³ containing nucleotides 134–614 of the mouse *Mas* gene removed the 5' part of *Mas* and truncated the YAC to 300 kb (YAC-T1/P). *Mas* is thought to be imprinted in a developmental and tissue-specific manner²⁴, but we have shown that *Mas* is not imprinted in laboratory mice²⁵. Region 2 was deleted in two steps: a 4-kb *Bam*HI–*Pac*I fragment containing region 2 was replaced by the yeast *URA3* gene, then *URA3* was removed by homologous recombination with a 2.3-kb linear fragment containing region-2 flanking sequences. YAC-T1/PΔR2 thus contained a 4-kb deletion eliminating all the CpG-rich sequences of region 2 but leaving splice junctions of the flanking second and third *Igf2r* exons.

Transgenic mice. YAC DNA was isolated for microinjection by preparative pulsed-field gel electrophoresis (PFGE) using 0.5% MP agarose (Boehringer Mannheim) and DNA concentrated by standard electrophoresis into 4% Nusieve GTG agarose (FMC Corporation, Philadelphia, PA, USA). Agarose was removed by Gelase (Epicenter). The size and concentration of the isolated YAC

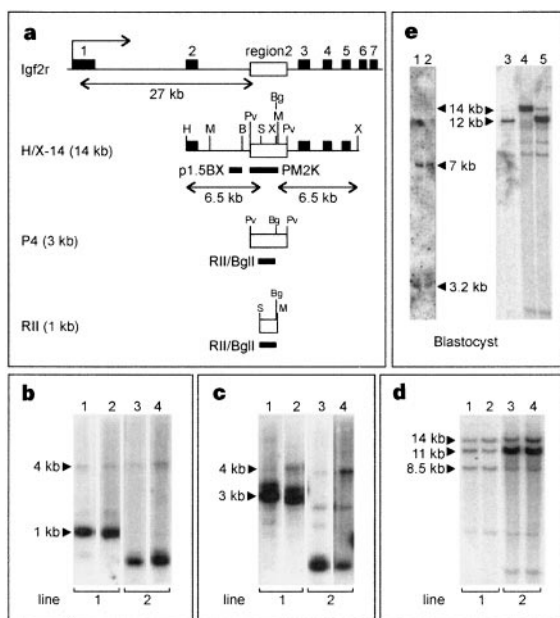


Figure 5 Methylation of region 2 on short transgenes. **a**, The mouse *Igf2r* locus and short transgenes carrying region 2. H/X-14 (a 14-kb *Hind*III/*Xho*I/*Xho*I fragment spanning exons 2–5); P4 (a 3-kb *Pvu*II–*Pvu*II fragment containing only region 2); RII (a 1-kb *Sfu*I–*Mlu*I fragment from the central part of region 2 that contains direct repeats¹⁴). H, *Hind*III; S, *Sfu*I; M, *Mlu*I; P, *Pac*I; B, *Bam*HI; Bg, *Bgl*II, Pv, *Pvu*II, X, *Xho*I. **b**, Tail DNA digested with *Bgl*II and *Hpa*II and blots hybridized with pRII/BglI. The transgene-specific 1-kb band is hemizygous and is not cut by *Hpa*II in line 1, but is cut in line 2. Maternal transmission, lanes 1 and 3; paternal transmission, lanes 2 and 4. The intensity of the 4.0-kb endogenous band is reduced by 50% by *Hpa*II. **c**, P4 transgenes analysed as in **b**. The transgene-specific 3-kb band is not cut by *Hpa*II in line 1, but is cut in line 2. Maternal transmission, lanes 1 and 3; paternal transmission, lanes 2 and 4. **d**, H/X-14 transgenes (line 1: 2–3 copies, line 2: 60–80 copies). The transgene-specific 14-kb fragment is cut to 11 kb when *Mlu*I is unmethylated. Maternal transmission and paternal transmission blots were hybridized with p1.5BX. The 8.5-kb endogenous band is the flanking *Bam*HI fragment upstream of region 2. The single tissue analysed in **b–d** is representative of a range of adult organs and E13.5 embryonic tissue. **e**, Methylation in blastocysts of line-2 H/X-14 transgene after maternal inheritance. In blastocysts, an additional *Mlu*I site upstream of region 2 in intron 2 is unmethylated on both parental alleles, so a 7-kb fragment will result if the *Mlu*I site inside region 2 is also unmethylated. Lanes 1 and 2, duplicate samples of 150 blastocysts digested with *Bam*HI and *Mlu*I hybridized with probe PM2K. Only a 7-kb *Mlu*I–*Mlu*I fragment is seen, indicating hypomethylation at the region 2 internal *Mlu*I site. This fragment is reduced to 3.2 kb by *Bam*HI (the *Bam*HI digest was incomplete). Lane 3, 230 blastocysts digested with *Bam*HI and *Sfu*I hybridized with probe PM2K. The 14-kb fragment is cut to 11 kb when *Sfu*I is unmethylated. Lanes 4 and 5, control digest with 0.1 μg adult spleen DNA from a maternally inherited H/X-14 transgene. The *Sfu*I site on the 14-kb *Bam*HI transgene-specific fragment remains hypomethylated in this tissue.

DNA was checked (Fig. 2a), dialysed (0.2 mM EDTA, 7.5 mM Tris-Cl, pH 7.5, 100 mM NaCl) by floating filter (Millipore VMWP04700), diluted and injected at $1 \text{ ng } \mu\text{l}^{-1}$ as described²⁶. 503 zygotes (C57Bl6/CBA-F1) were injected with YAC-T1/P, seventeen pups developed, five were transgenic and four transmitted the transgene. Microinjection of 1,006 zygotes with YAC-T1/PAR2 DNA yielded 99 offspring, of which nine were transgenic; three lines were bred. YAC transgenes were genotyped by PCR. The acentric YAC vector arm was identified using the *LYS2* gene (463 bp, primer pair TTGGACAATGGCGAGGAT and ACGCTGTGTTTCAGTGGTACC). The centric YAC arm was identified using the *TRP1* gene (290 bp; primer pair TAAGTATTGTTGTGCACTTGCCTGC and GTATTTATATACTAAGCTGCCGGCGG). The integrity of the integrated YACs was assayed by probes p15 (5-kb *EcoRI* fragment containing the *Igf2r* promoter and exon 1), p1.5BX (a 1.5-kb *BAMHI*-*XbaI* fragment outside region 2 but within intron 2). Standard techniques were used for the experiments shown in Fig. 5. Other probes used were: PM2K (a 2-kb *PvuII*-*MluI* fragment within region 2), RII/BglI (a 0.8-kb *SfiI*-*BglI* fragment within region 2).

Blastocyst DNA. Blastocysts from hormone-primed²⁶ transgenic females were washed in M2 medium, embedded in 0.5% low-melting-point agarose, incubated for 24 h at 50 °C in 0.5 M EDTA, pH 8, 1% sodium lauryl sarkosine, 0.5 mg ml^{-1} proteinase K, and digested with restriction enzymes.

Expression analysis. For northern blots and RNase protection assay, 5–10 mg total RNA were used²⁷. Riboprobe 1 (175 bp), 5' [nucleotides 117–82 of mouse *Igf2r* exon 1, joined to nucleotides 2,572–2,472 of mouse Plasminogen terminal exon]3', protected a 135-bp fragment specific for tagged YAC-*Igf2r* mRNA (Figs 3, 4). Riboprobe 2 (190 bp), 5' (nt 117–82 of mouse *Igf2r* joined to nt 2,105–2,238 of Plasminogen]3', protected a 142-bp fragment specific to tagged YAC-*Igf2r* AS RNA (and a fragment of 35 bp not resolved in the gels in Figs 3 and 4). Riboprobe 3 (243 bp) is a *SaII*-*SmaI* fragment containing 20 bp of exon 1 and 194 bp of intron 1, and protects a 214-bp fragment specific for wild-type AS RNA (Fig. 3c). In Fig. 4a, endogenous *Plg* mRNA, expressed in fetal liver, was also detected by riboprobe 1 as a 100-bp fragment. Products were analysed on 8% polyacrylamide/46% urea gels.

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Activation of ATP P2X receptors elicits glutamate release from sensory neuron synapses

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Painful stimuli to the skin initiate action potentials in the peripheral terminals of dorsal root ganglion (DRG) neurons. These action potentials propagate to DRG central terminals in the dorsal horn of the spinal cord, evoking release of excitatory transmitters such as glutamate onto postsynaptic dorsal horn neurons. P2X receptors, a family of ligand-gated ion channels^{1,2} activated by the endogenous ligand ATP, are highly expressed by DRG neurons^{3–5}. Immunoreactivity to P2X receptors has been identified in the dorsal horn superficial laminae associated with nociceptive DRG central terminals⁵, suggesting the presence of presynaptic P2X receptors. Here we have used a DRG–dorsal horn co-culture system to show that P2X receptors are localized at presynaptic sites on DRG neurons; that activation of these receptors results in increased frequency of spontaneous glutamate release; and that activation of P2X receptors at or near presynaptic DRG nerve terminals elicits action potentials that cause evoked glutamate release. Thus activation of P2X receptors at DRG central terminals can modify sensory signal throughput, and might even initiate sensory signals at central synapses without direct peripheral input. This putative central modulation and generation of sensory signals may be associated with physiological and pathological pain sensation, making presynaptic P2X receptors a possible target for pain therapy.

As occurs *in vivo*, glutamatergic synapses were formed between DRG and dorsal horn neurons in micro-island co-cultures, as determined by paired cell recording of evoked excitatory postsynaptic currents (EPSCs; not shown). By recording the response of dorsal horn neurons to focal application of ATP, we tested whether activation of P2X receptors on DRG neurons induced glutamate release. To limit ATP action to P2X receptors at or near DRG nerve terminals, DRG cell bodies and their proximal neurites were removed using a suction pipette before recording from a dorsal horn neuron (Fig. 1a). In other cases, when DRGs were grown as explants with dissociated dorsal horn neurons, DRG explants were removed.

By using a micro-island preparation similar to that shown in Fig. 1a (right), recordings were made from dorsal horn neurons while 200 μM ATP was focally puffed onto the dorsal horn neurites with their associated DRG nerve terminals for 100 ms. All experiments were conducted in the presence of 500 nM tetrodotoxin (TTX), which completely blocked the generation of action potentials by

dorsal horn neurons ($n = 5$, data not shown), but not by DRG neurons ($n = 15$, data not shown), owing to the presence of TTX-resistant Na^+ channels⁶. ATP evoked large, transient, sometimes repetitive inward currents with amplitudes often 20-fold larger than miniature EPSC (mEPSC) amplitudes recorded from the same neurons (Fig. 1b). This response to ATP desensitized, requiring more than 5 min for full recovery. Similar results were obtained in seven out of eight cells tested. Subsequent pharmacological tests of ATP-evoked responses were performed with a minimum of 8 min between ATP applications. ATP-evoked transient currents recorded from dorsal horn neurons were blocked when 100 μM CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) was present in the bathing solution (Fig. 1b), indicating that ATP caused glutamate to be released onto the dorsal horn neurons. Addition of 50 μM PPADS (pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid) blocked ATP-evoked glutamate release, suggesting that the response was mediated by ionotropic P2X receptors⁷. Block of glutamate release by PPADS partly recovered after wash (Fig. 1b). Similarly, 50 μM PPADS blocked ATP-evoked currents recorded directly from the DRG soma, with recovery being slow and incomplete (Fig. 1c).

Because P2X receptors are usually found to be Ca^{2+} -permeable^{8–11}, we examined whether Ca^{2+} entry through the receptors caused ATP-evoked glutamate release. ATP-evoked currents recorded from

dorsal horn neurons were blocked when Ca^{2+} was removed from the bathing solution, showing that Ca^{2+} entry was required (Fig. 1b). Surprisingly, when the voltage-gated Ca^{2+} -channel blocker La^{3+} was present¹² at 30 μM , ATP-evoked glutamate release was blocked (Fig. 1b). Similar results were obtained in two other DRG–dorsal horn micro-island preparations and three DRG explant preparations. However, La^{3+} (30 μM) did not block current flow through P2X receptors expressed on DRG neurons (Fig. 1c). These results suggest that ATP-evoked glutamate release from DRG nerve terminals involves activation of both P2X receptors and voltage-gated Ca^{2+} channels.

Two lines of evidence show that the site of ATP action is on P2X receptors expressed on DRG neurons, not dorsal horn neurons. First, although ATP evoked glutamate release from DRG nerve terminals onto dorsal horn neurons in most experiments using DRG–dorsal horn co-cultures, no such responses could be recorded using dorsal horn monocultures in the presence of 500 nM TTX ($n = 12$ data not shown). Second, when P2X receptor-mediated whole-cell currents were occasionally induced directly from dorsal horn neurons, they were non-desensitizing¹³. In contrast, the strong desensitization of ATP-evoked glutamate release (Fig. 1b) is consistent with the desensitization of ATP-evoked whole-cell currents measured directly from DRG neurons (Fig. 1c; $n = 3$).

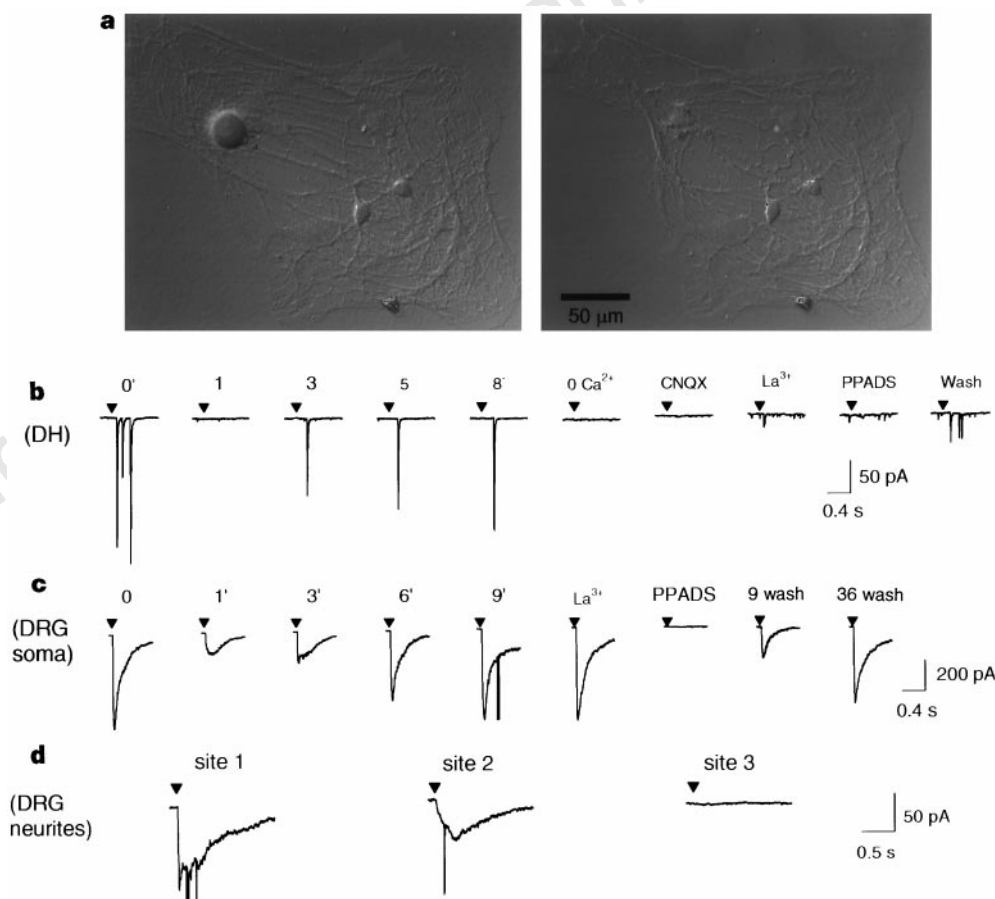


Figure 1 Glutamate release and TTX-resistant action-potential-like currents evoked by focal application of ATP onto DRG neurites. **a**, Left, micro-island co-culture of a DRG neurons (large cell) and two dorsal horn neurons (small cells). DRG neurites form a network over the whole micro-island. Dorsal horn neurites can also be seen. Right, DRG soma and some proximal neurites were removed. **b**, ATP (200 μM) puffed onto a dorsal horn neurite with associated DRG terminals evoked glutamate release. The effect shows desensitization with slow recovery. Numbers above the traces indicate the time interval (min) since the previous ATP application. Evoked responses, measured with 8 min between ATP applications, were blocked by 0 Ca^{2+} , 100 μM CNQX, 30 μM La^{3+} and 50 μM PPADS. **c**, P2X

receptor-mediated currents recorded from a DRG soma when 200 μM ATP was puffed directly onto it. Numbers above the traces indicate the time interval (min) since the previous ATP application. The currents show desensitization, slow recovery, little sensitivity to 30 μM La^{3+} , and complete block by 50 μM PPADS with slow and incomplete recovery. Numbers before wash indicate time (min) spent washing away PPADS. **d**, Puffing 200 μM ATP on DRG neurites 150 μm (site 1), 112 μm (site 2) and 75 μm (site 3) from the soma elicited action-potential-like current spikes recorded from the DRG soma. In all experiments, puff duration was 100 ms. Arrowheads indicate the ATP application start time.

The large size of the currents caused by ATP-evoked glutamate release, and the involvement of voltage-gated Ca^{2+} channels, suggests that the currents resulted from the synchronous release of multiple vesicles of glutamate. This would occur if focal activation of P2X receptors at or near DRG terminals depolarized neuritic membrane sufficiently to evoke action potentials. To test this possibility, we puffed ATP at different sites along neurites of DRG neurons while voltage clamping their soma at -70 mV. ATP-induced currents at three different sites on DRG neurites 75–150 μm away from the DRG soma are shown in Fig. 1d. At sites 1 and 2, neurites crossed over that appeared to originate from dorsal horn neurons; site 3 did not have a visible dorsal horn neurite crossing over. Puffing 200 μM ATP at sites 1 and 2 evoked inward currents with different amplitudes, which were accompanied by current transients reflecting underlying action potentials; at site 3, ATP evoked little detectable current and no such current spikes. Similar results were obtained in two other experiments. These results indicate that activation of P2X receptors on DRG neurites elicits TTX-resistant action potentials at sites distant from the soma that may account for the large ATP-evoked glutamate release events shown in Fig. 1b.

We tested whether activation of P2X receptors after focal ATP application was localized to discrete regions near dorsal horn neurites, or whether diffusion of ATP caused a more global activation of P2X receptors on nearby neurites of DRG neurons. A recording from a dorsal horn neuron in a culture preparation similar to that of Fig. 1a (right) is shown in Fig. 2a. ATP was applied to two sites 50 μm apart; site A responded, but site B did not. Furthermore, when two different ATP-sensitive locations were tested, no cross-desensitization occurred (Fig. 2b). ATP-evoked responses recorded from a dorsal horn neuron in a DRG explant culture when ATP was alternatively puffed at two separate sites 50 μm from the dorsal horn soma are shown in Fig. 2b. ATP evoked glutamate release at each site, and both were desensitized and unable to respond when ATP was subsequently applied within 1 min. A slow and gradual recovery from desensitization was observed at both sites, but no cross-desensitization was seen. These results are consistent with the idea that ATP applied focally activates P2X

receptors near DRG nerve terminals, and does not simply stimulate a large area of DRG neuritic membrane.

Synchronous release of glutamate resulting from activation of P2X receptors at or near DRG nerve terminals requires activation of voltage-gated Ca^{2+} channels, probably driven by TTX-resistant action potentials. However, P2X receptors expressed by DRG neurons are themselves likely to be permeable to Ca^{2+} (ref. 11, but see ref. 14). If P2X receptors are expressed on presynaptic terminals, they should provide a direct route of Ca^{2+} entry, resulting in an increase in spontaneous release of glutamate, as has been shown for presynaptic Ca^{2+} -permeable nicotinic receptors¹⁵. To examine this, we first had to block TTX-resistant action potentials. Voltage-activated inward currents in DRG neurons in the presence of 500 nM TTX are shown in Fig. 3a, b. After block of voltage-gated Ca^{2+} currents by 30 μM La^{3+} , there was a remaining transient current, thought to be mediated by TTX-resistant Na^+ channels known to be expressed by DRG neurons⁶ (Fig. 3a). TTX-resistant Na^+ currents were blocked by 10 mM lidocaine (Fig. 3b) in a way that was not use-dependent. Furthermore, voltage-gated Ca^{2+} currents were also blocked by 10 mM lidocaine (Fig. 3b), such that little Ca^{2+} current remained at the end of an 80-ms voltage step (Fig. 3b). Consistent with this action of lidocaine, ATP-evoked synchronous glutamate release was abolished in the presence of 10 mM lidocaine (Fig. 3c; $n = 4$).

We examined the effect of ATP on spontaneous glutamate release by measuring mEPSCs in bathing solution containing 10 mM lidocaine (Fig. 4). Bath application of 100 μM ATP increased mEPSC frequency by 200 to 700% ($444 \pm 87\%$) over control values in all seven cells tested (Fig. 4b–d). Application of 50 μM PPADS prevented ATP-induced enhancement of mEPSC frequency (Fig. 4c, d; $n = 3$). The increase in mEPSC frequency after ATP application often caused current summation of several concurrent mEPSCs (Fig. 4b), which may have caused the small but significant ($124.29 \pm 7.82\%$) increase in measured mean mEPSC amplitude. A possible effect of ATP on postsynaptic glutamate receptors¹⁶ is less likely to account for the increased mEPSC amplitude because glutamate-evoked whole-cell currents were not significantly

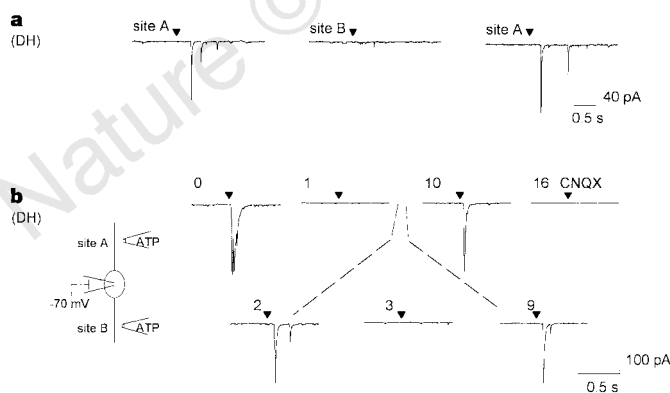


Figure 2 Response to focal application of ATP at different sites on neurites. **a**, ATP (200 μM) puffed on a dorsal horn neurite at two sites 50 μm apart. ATP evoked glutamate release at site A and had no effect at site B. **b**, No cross-desensitization between the ATP-sensitive sites A and B (each 50 μm from the dorsal horn soma) although desensitization occurred at each site. In all experiments, recordings were made on dorsal horn neurons in DRG–dorsal horn micro-island co-cultures or DRG–dorsal horn explant cultures with DRG soma and their proximal neurites removed. ATP (200 μM) was applied for 100 ms. Arrowheads indicate ATP application start time. Numbers above the traces indicate the actual time sequence (min) of experiments.

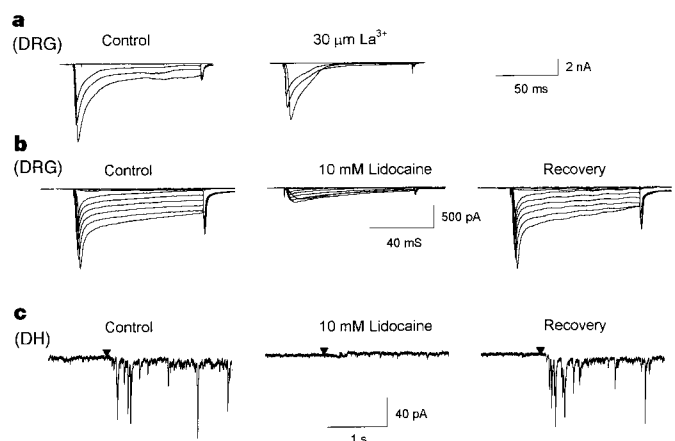


Figure 3 Inhibition of TTX-resistant Na^+ channels and ATP-evoked glutamate release by 10 mM lidocaine. **a**, Effect of La^{3+} on steady-state voltage-gated current. In the presence of 500 nM TTX, voltage steps from -70 to $+10$ mV in steps of 20 mV activated voltage-gated inward currents on a DRG neuron. The steady-state current, primarily resulting from voltage-gated Ca^{2+} channels, was blocked by 30 μM La^{3+} . The remaining early current was TTX-resistant Na^+ current⁶. **b**, Lidocaine (10 mM) block of TTX-resistant Na^+ currents and much of the voltage-gated Ca^{2+} currents. Voltage steps from -70 to $+40$ mV were applied in steps of 10 mV. **c**, Block of ATP-evoked glutamate release by 10 mM lidocaine. Recordings were made on dorsal horn neurons grown on DRG–dorsal horn explant co-cultures with DRGs removed. ATP (200 μM) was puffed on the dorsal horn neurites for 100 ms. Arrowheads indicate the ATP application start time.

affected by co-application of glutamate with 100 μ M ATP ($n = 4$), and were not inhibited by 50 μ M PPADS (Fig. 4f; $n = 4$). In different cells, 100 μ M α,β -methylene-ATP ($\alpha\beta$ m-ATP) resulted in an increase in mEPSC frequency to near 250% of control (243 ± 24 , $n = 5$), without a change in mEPSC amplitude; this was followed by recovery back to baseline following washout of $\alpha\beta$ m-ATP (Fig. 4d). We used $\alpha\beta$ m-ATP because it is a selective agonist for P2X receptors over P2Y receptors¹, and at 100 μ M it has little direct effect on dorsal horn neurons¹³. The experiments with $\alpha\beta$ m-ATP were performed using micro-islands with several dorsal horn and DRG neurons, whereas experiments with ATP alone were performed using micro-islands with a single dorsal horn neuron and one or a few DRG neurons. mEPSCs due to glutamate release from ATP-insensitive dorsal horn nerve terminals may cause an underestimation of the per cent change in mEPSC frequency in experiment using $\alpha\beta$ m-ATP.

The increase in mEPSC frequency after activation of P2X receptors is dependent on Ca^{2+} entry because low extracellular Ca^{2+} largely prevented the ATP-induced increase of mEPSC frequency (Fig. 4d; $n = 5$). Even though voltage-gated Ca^{2+} channels are strongly blocked in the presence of 10 mM lidocaine (Fig. 3b), ATP action on presynaptic P2X receptors depolarizes membrane potential and could activate residual voltage-gated Ca^{2+} entry. Therefore, ATP-induced increase in mEPSC frequency was further tested when voltage-gated Ca^{2+} channels were blocked with 30 μ M La^{3+} . In the continuous presence of La^{3+} , ATP caused a significant increase in mEPSC frequency of $226 \pm 34\%$, with no change of mEPSC amplitude (Fig. 4d). These recordings were made under the same culture conditions as those with $\alpha\beta$ m-ATP, and the increase in mEPSC frequency is similar. Thus Ca^{2+} entry through P2X receptors is sufficient to cause a large increase of spontaneous glutamate release, suggesting that P2X receptors are located at presynaptic nerve terminals.

In contrast to the ATP-evoked synchronous release of glutamate induced by brief focal application of ATP (Figs 1 and 2), the increase in mEPSC frequency following activation of P2X receptors showed only slight desensitization with prolonged ATP application (Fig. 4c). Prolonged whole-cell application of ATP to DRG neurons ($n = 3$) resulted in large inward currents with strong desensitization over the first 20 s of ATP application (Fig. 4e). The desensitization then slowed and a steady-state current was reached at about 1 min which lasted to the end of the 5-min period of ATP application. Thus ATP-evoked glutamate release (Figs 1, 2) occurs when P2X receptor currents are strong enough to initiate action potentials (Fig. 1b, d). The increase of spontaneous glutamate release during prolonged ATP application (Fig. 4b–d) may be due to the residual steady-state ATP current.

Release of endogenous ATP into the superficial laminae of the dorsal horn^{13,17} in the vicinity of the nociceptive DRG nerve terminals is expected to activate presynaptic P2X receptors there. This may be an important mechanism for controlling sensory transmission at the first sensory synapse by modulating the amount of transmitter released. Other presynaptic ionotropic receptors have been shown to regulate transmitter release at central synapses^{15,18}. Activation of presynaptic NMDA (*N*-methyl-D-aspartate) receptors on nociceptive primary afferent nerve terminals has recently been shown to induce release of substance P into the dorsal horn of the spinal cord¹⁹. Here we show that activation of presynaptic P2X receptors increases quantal glutamate release and, perhaps more importantly, evokes action potentials that drive large evoked synchronous release of multiple quanta of glutamate. It will be interesting to test whether activation of presynaptic P2X receptors releases substance P as well as glutamate. Our studies showing the synaptic consequences of action potentials evoked at or near the presynaptic nerve terminals of DRGs are complementary to the

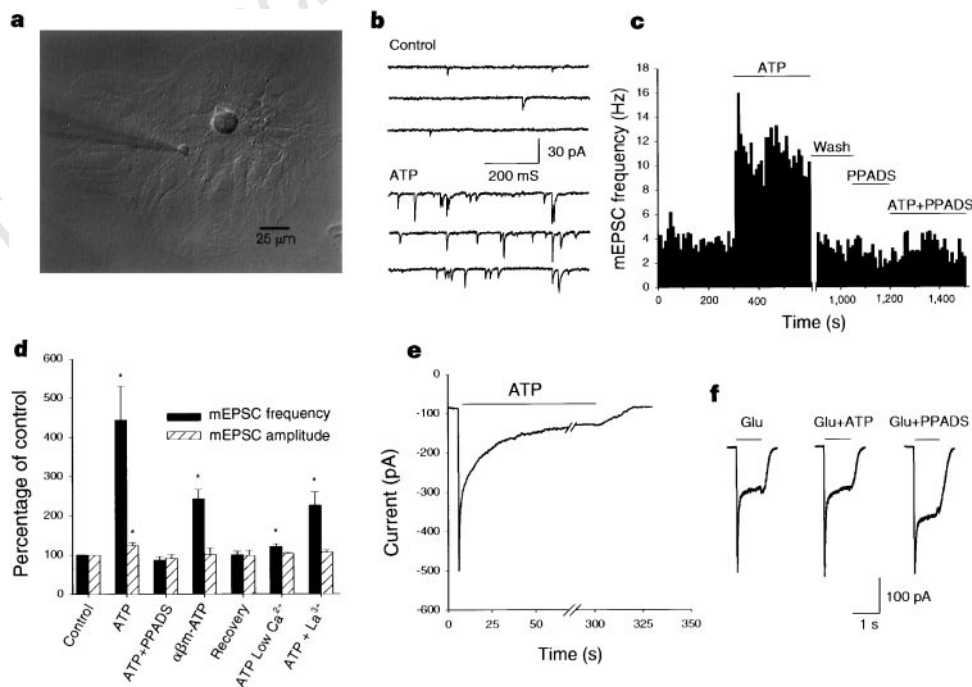


Figure 4 Increase in spontaneous glutamate release following activation of presynaptic P2X receptors on DRG nerve terminals. **a**, A micro-island cultures with a single DRG (large cell) and a single dorsal horn neuron (small cell). **b**, Sample traces of mEPSCs recorded from a dorsal horn neuron before and during 100 μ M ATP application in a bathing solution containing 10 mM lidocaine. **c**, The histogram of mEPSC frequency before, during 100 μ M ATP application, and after addition of 50 μ M PPADS. The time bin is 10 s. **d**, A summary of the change of mEPSC frequency and amplitude following different drug conditions. Conditions tested include 100 μ M ATP in the absence ($n = 7$) and presence ($n = 3$) of 50 μ M

PPADS, 100 μ M $\alpha\beta$ m-ATP ($n = 5$) and following wash of $\alpha\beta$ m-ATP ($n = 5$), ATP in low Ca^{2+} bathing solution in which Ca^{2+} was not added ($n = 5$), and ATP plus 30 μ M La^{3+} ($n = 4$). mEPSC frequency and amplitude were averaged for each cell over the first 1 min of ATP and $\alpha\beta$ m-ATP application. Values represent mean $\pm$ s.e.m. * $P < 0.05$. **e**, Whole-cell current recorded from a DRG neuron in response to a 5-min application of 100 μ M ATP in the presence of 10 mM lidocaine. **f**, Whole-cell currents recorded from dorsal horn neurons evoked by 100 μ M glutamate alone, coapplied with 100 μ M ATP ($n = 4$) and with 50 μ M PPADS ($n = 4$). PPADS was preapplied for 3 min.

recent observation that activation of P2X receptors on neurites of identified nociceptors induces firing of action potentials²⁰. In that study²⁰ it was suggested that P2X receptors localized at peripheral sensory nerve terminals provide a mechanism for sensing nociceptive stimulation. Thus it is possible that P2X receptors expressed on both the peripheral terminals and central presynaptic terminals are capable of modulating or generating nociceptive signalling. □

Methods

Cell cultures. Monocultures of dorsal horn neurons were prepared as described²¹. For DRG–dorsal horn co-cultures, dorsal horn neurons were isolated from rat embryos aged 16 days (E16) *in utero*, exposed to 0.25% trypsin for 20 min and dissociated. Similarly, DRGs were isolated separately from E16 embryos, exposed to trypsin and dissociated. Dorsal horn and DRG neurons were plated on glass coverslips previously prepared with a monolayer of rat cortical astrocytes. At the time of plating, 2.5S NGF (10 ng ml⁻¹) and 5-fluoro-2'-deoxyuridine (10 µM) were added, and 2.5S NGF was added once every week when cells were fed with fresh media. For dorsal horn plus DRG explant cultures, conditions were the same except that the ganglia were isolated and plated directly on the centre of the glass coverslips. Micro-island cultures²² were prepared as follows. Coverslips were precoated with PDL then dipped in 0.5% agarose (type I, low EEP; Sigma) and allowed to dry for 1 h. Once dry, the dishes were sprayed with rat-tail collagen (2 mg ml⁻¹ in a 0.2% acetic acid solution) using an atomizer. The dishes were sterilized by ultraviolet irradiation for 2 h, then plated with astrocytes. After 3–7 days, neurons were plated on top of the astrocytes. The plating density was usually 10,000–30,000 dorsal horn neurons per dish, and 30,000–50,000 DRG neurons per dish. Cultures at 2–4 weeks were used for experiments. In some experiments, DRG cell bodies were removed for co-culture dishes before experiments, and dorsal horn neurons and remaining DRG fibres stayed healthy for at least 2 h.

Electrophysiology recordings. Standard bathing solution contained (in mM): 145 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES, 5.5 D-glucose, and 5 × 10⁻⁴ TTX, pH 7.3 with NaOH, 325 mOsm with sucrose, flow rate of 1 ml min⁻¹, and room temperature. Bicuculline (10 µM) and strychnine (5 µM) were also present. Dorsal horn neurons were voltage-clamped²¹ at -70 mV in perforated whole-cell patch configuration (Axopatch 200) with electrodes containing internal solution (in mM): 75 Cs₂SO₄, 10 CsCl, 0.1 CaCl₂, 10 HEPES and 400 µg ml⁻¹ amphotericin B, pH 7.3 with CsOH and 315–325 mOsm with sucrose. For experiments on ATP-evoked glutamate release, 200 µM ATP was puffed for 100 ms with puff pipettes of 1.5–2 µm diameter at 3.5–6.5 p.s.i. Recovery periods (4–8 min) were allowed between ATP applications except when otherwise specified. For experiments testing pharmacology of ATP-evoked currents, cells were rested in normal bathing solution for ~8 min to allow recovery from desensitization caused by previous trials. CNQX (100 µM), La³⁺ (30 µM) and PPADS (50 µM) were preapplied to the bathing solution for 2–4 min. Effects of P2X receptor activation on spontaneous glutamate release from presynaptic terminals of DRG neurons were determined by measuring the effects of 100 µM ATP and 100 µM αβm-ATP on mEPSCs recorded from dorsal horn neurons. Cultures were perfused with standard bathing solution plus 10 mM lidocaine. mEPSCs were recorded from dorsal horn neurons voltage-clamped at -70 mV in perforated patch configuration²¹. After 5 min recording of control mEPSCs, 100 µM ATP or αβm-ATP was bath-applied for 5 min and mEPSCs were continuously recorded. Effects of PPADS were tested following wash of ATP for several minutes to allow mEPSCs to return to the basal control conditions. After 3–5 min of pre-applying 50 µM PPADS, ATP and 50 µM PPADS were then coapplied and mEPSCs recorded. In experiments measuring mEPSCs in low extracellular Ca²⁺ concentrations, the bathing solution was the same as the standard bathing solution except that Ca²⁺ was not added. In experiments with La³⁺, 30 µM La³⁺ was present at all times in bathing solution and ATP was applied with 30 µM La³⁺. Thus, although basal mEPSC frequency was found to be affected by 30 µM La³⁺, ATP always increased mEPSC frequency above that in La³⁺. mEPSC sampling and data analysis were done as described²¹. Electrophysiological recordings from DRG neurons were made using conventional whole-cell voltage clamps at -70 mV with patch electrodes containing intracellular solution of (in mM): 130 Cs-gluconate, 10 CsCl, 11 EGTA, 1 CaCl₂, 10 HEPES, 20 TEA, 2 ATP-Mg, 305 mOsm, pH 7.2. A paired Wilcoxon test was

used for statistical analysis of the mean mEPSC frequency and amplitude under different experimental conditions.

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Molecular basis of agonism and antagonism in the oestrogen receptor

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Oestrogens are involved in the growth, development and homeostasis of a number of tissues¹. The physiological effects of these steroids are mediated by a ligand-inducible nuclear transcription factor, the oestrogen receptor (ER)². Hormone binding to the ligand-binding domain (LBD) of the ER initiates a series of molecular events culminating in the activation or repression of

target genes. Transcriptional regulation arises from the direct interaction of the ER with components of the cellular transcription machinery^{3,4}. Here we report the crystal structures of the LBD of ER in complex with the endogenous oestrogen, 17 β -oestradiol, and the selective antagonist raloxifene⁵, at resolutions of 3.1 and 2.6 Å, respectively. The structures provide a molecular basis for the distinctive pharmacophore of the ER and its catholic binding properties. Agonist and antagonist bind at the same site within the core of the LBD but demonstrate different binding modes. In addition, each class of ligand induces a distinct conformation in the transactivation domain of the LBD, providing structural evidence of the mechanism of antagonism.

The structure of the complex between ER's LBD and the antagonist raloxifene (RAL) was determined by conventional multiple isomorphous replacement in combination with multicrystal averaging, and was subsequently used as a phasing model in molecular replacement to solve the structure of the complex of the LBD

with 17 β -oestradiol (E₂) (see Methods and Table 1). The overall architecture of the ER LBD (helices H3–H12) is similar to that seen in the crystal structures of other nuclear receptor LBDs^{6–8}, and emphasizes the universal nature of this fold within this receptor superfamily⁹. The LBD is folded into a three-layered antiparallel α -helical sandwich comprising a central core layer of three helices (H5/6, H9 and H10) sandwiched between two additional layers of helices (H1–4 and H7, H8, H11). This helical arrangement creates a 'wedge-shaped' molecular scaffold that maintains a sizeable ligand-binding cavity at the narrower end of the domain. The remaining secondary structural elements, a small two-stranded antiparallel β -sheet (S1 and S2) and H12, are located at this ligand-binding portion of the molecule, and flank the main three-layered motif (Fig. 1a).

The ER LBDs are arranged as non-crystallographic dimers within both the E₂ and RAL complex crystals in a manner consistent with both the oligomeric state of liganded ER in solution¹⁰ and previous

Table 1 Data collection, phase determination and refinement statistics

	ER RAL	ER E ₂	ER RAL derivatives		
			PCMBs-1 (4 mM, 5 day)	PCMBs-2 (4 mM, 14 day)	KAu(CN) ₂ (4 mM, 2 day)
Resolution (Å)	25–2.6	20–3.1	20–3	20–3	20–3.6
Unique reflections	15,433	33,981	10,335	9,316	5,835
Completeness (%)	95.7	99.1	97.6	89.0	94.2
Multiplicity	4.5	2.5	4	3.1	2.5
R _{sym} (I)†	8.0	10.0	8.1	9.2	7.0
R _{iso} †			16.9	20.7	13.7
Phasing power (centric/acentric)‡			1.22/1.88	1.23/2.02	0.71/0.94
R _{Cullis} (centric/acentric)§			0.75/0.68	0.76/0.66	0.90/0.85
Refinement					
Reflections used (R _{free} set)	13,868 (1,565)	30,583 (3,398)			
R _{cryst} (R _{free})	21.9 (29.9)	21.8 (25.1)			
Protein (solvent) atoms	3,633 (100)	11,382 (114)			
% A,B,L (a,b,l,p)¶	94.2 (5.8)	94.2 (5.8)			
R.m.s.d. bond lengths/angles (Å)#	0.016/0.035	0.011/0.039			
R.m.s.d. n.c.s. protein (Å)*	0.66	0.07			
R.m.s.d. n.c.s. B (Å ²)**	7.9	1.15			

*R_{sym} (I) = 100 × $\sum_i \sum_j |I_i - I_j| / \sum_i \sum_j I_i$, where I is the observed intensity, I_j is the average intensity of multiple observations of symmetry-related reflections.

†R_{iso} = $\sum_i |F_{PH}| - |F_P| / \sum_i |F_{PH}|$, where |F_P| is the protein structure factor amplitude and |F_{PH}| is the heavy-atom derivative structure factor amplitude.

‡Phasing power for centric and acentric reflections = r.m.s. (|F_H|/E), where F_H is the heavy atom structure factor amplitude and E is the residual lack of closure error.

§R_{Cullis} = $\sum_i |E| / \sum_i |F_{PH}| - |F_P|$ for centric and acentric reflections. Figure of merit was 0.48 for acentric reflections and 0.67 for centric reflections (20–3 Å).

||R_{cryst} = 100 × $\sum_i |F_{PH}| - |F_P| / \sum_i |F_{PH}|$; R_{free} is the same as R_{cryst} but was calculated using a separate validation set of reflections that was excluded from the refinement process.

¶Percentage of residues located in most favoured (additional) regions of the Ramachandran plot as determined by PROCHECK²⁰.

#R.m.s. deviation in bond length and angle distances from Engh and Huber ideal values.

*Root mean squared distance between all non-crystallographic symmetry (n.c.s.) related protein atom positions.

**R.m.s. difference between all n.c.s.-related atomic temperature factors.

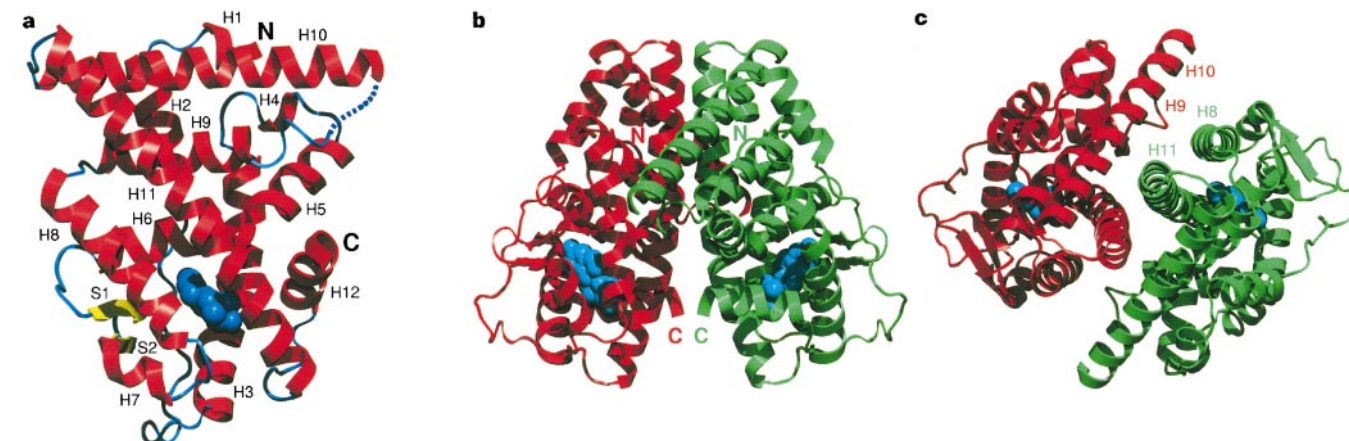


Figure 1 Ribbon representations of the ER- α LBD. **a**, The ER- α LBD indicating the locations of the secondary structural elements. α and 3_{10} helices (H) are coloured red, extended regions (S) are yellow, and coil regions are blue. All secondary structural elements have been numbered in keeping with the nomenclature that has been established for other nuclear receptor LBDs. The monomer is displayed looking onto the dimerization face. The dotted line indicates the unmodelled

region between H9 and H10. **b**, ER- α LBD homodimer viewed perpendicular to the dimer axis. **c**, ER- α LBD homodimer viewed down the dimer axis. The component monomers are drawn in red and green. The N and C termini and the helices that are involved in the dimer interface are labelled. E₂ is coloured blue and depicted in space-filling form.

mutagenesis studies¹¹. All crystal forms of the liganded ER LBD obtained so far contain identical non-crystallographic dimers (data not shown). The overall homodimeric arrangement is the same in both the E₂ and RAL complexes, and is reminiscent of the crystallographic apo-retinoid-X receptor homodimer⁸. The dimer axis roughly coincides with the longest dimension of the LBD with each molecule tilted approximately 10° away from the two-fold axis. This symmetric 'head-to-head' arrangement locates the chain termini of each monomer on opposite sides of the dimer with the carboxy termini projecting towards the two-fold axis (Fig. 1b). The H8/H11 face of the monomers line up to form an extensive dimerization interface that encompasses about 15% (1,703 Å²) of each monomer's surface area. Contacts between the two molecules are made primarily through the H11 helices, which intertwine to form a rigid backbone, but also involve H8 from one monomer and parts of H9 and H10 from the neighbouring monomer (Fig. 1c).

The E₂ binding cavity is completely partitioned from the external environment and occupies a relatively large portion of the ER LBD's hydrophobic core (Fig. 1a). It is located at one end of the molecule and is formed by parts of H3 (Met 342 to Leu 354), H6 (Trp 383 to Arg 394), H8 and the preceding loop (Val 418 to Leu 428), H11 (Met 517 to Met 528), H12 (Leu 539 to His 547) and the S1/S2 hairpin (Leu 402 to Leu 410). Hormone recognition is achieved through a combination of specific hydrogen bonds and the complementarity of the binding cavity to E₂'s non-polar character (Fig. 2a,c). E₂ binds diagonally across the cavity between H11, H3 and H6 and adopts a low-energy conformation. The phenolic hydroxyl of the A-ring (O3; see Fig. 2c for atom numbering) nestles between H3 and H6 and makes direct hydrogen bonds to the carboxylate of Glu 353, the guanidinium group of Arg 394, and a water molecule. The 17-β hydroxyl (O17) of the D-ring makes a single hydrogen bond with His 524 in H11. The remainder of the molecule participates in a number of hydrophobic

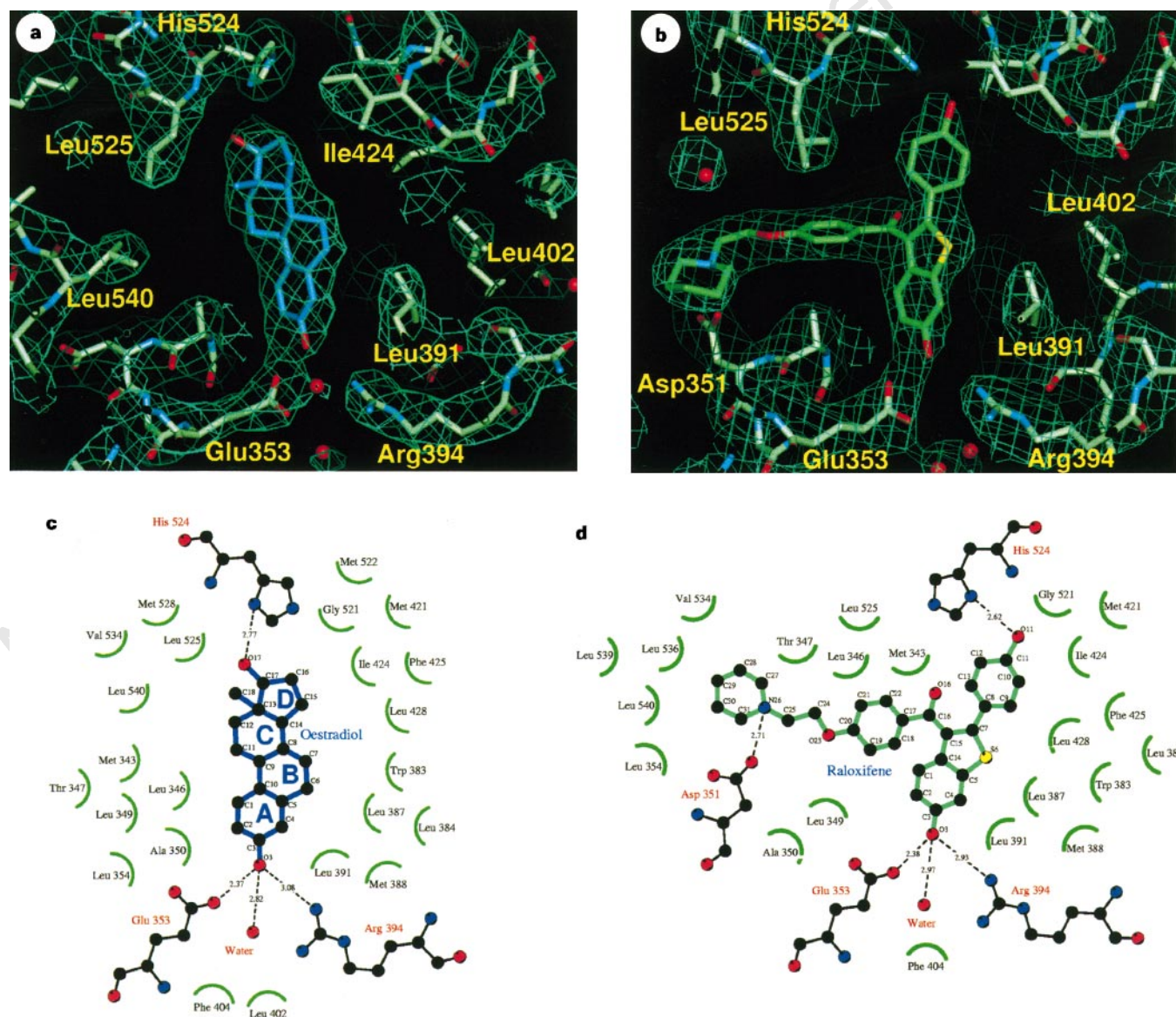


Figure 2 Agonist and antagonist binding modes. **a**, The 3.1-Å resolution, six-fold averaged electron-density map (using model phases) for the ER LBD-E₂ complex. **b**, The experimental, 2.6-Å resolution electron-density map for the ER LBD-RAL complex after DMMULTI multicrystal averaging. In both cases, the map is contoured at 1σ and overlaid on the final refined models. **c**, **d**, Schematic representation of the interactions made by E₂ (**c**) and RAL (**d**) within the binding

cavity. Residues that interact with ligand and/or line the cavity are shown in their approximate positions. Those that make direct hydrogen bonds are depicted in ball-and-stick style with broken lines between the interacting atoms. The hydrogen-bond distances shown are averaged between the six (E₂) or two (RAL) monomers. The atom names and ring nomenclature of E₂ are also given.

contacts that are concentrated over the A, A/B interface and D-rings. The A-ring, as well as the planar A/B-ring interface, is sandwiched between the side chains of Ala 350 and Leu 387 on its β face and Phe 404 on its α face. At the other end of the binding cavity, the D-ring makes non-polar contacts with Ile 424, Gly 521 and Leu 525. Although the cavity itself appears to be devoid of ordered water molecules, an extensive solvent channel runs from the A-ring hydroxyl's water ligand to the exterior of the LBD between H3 and H5/6. The combination of the specific polar and non-polar interactions account for the ability of ER to selectively recognize and bind E_2 with subnanomolar affinity over the large and varied range of endogenous steroids.

Extensive binding studies of E_2 analogues have provided a detailed description of the pharmacophore of ER¹². The ER is unique among the steroid receptors in its ability to embrace a wide variety of non-steroidal compounds. Although the 'pincer-like' arrangement around the A-ring imposes an absolute requirement on effective ligands to contain an aromatic ring, the remainder of the binding pocket can accept a number of different hydrophobic groups^{12,13}. This overall promiscuity can be attributed to the size of the cavity, which has a probe accessible volume (450 Å³) nearly twice that of E_2 's molecular volume (245 Å³). The length and breadth of the E_2 skeleton is well matched by the receptor, but there are large unoccupied cavities opposite the α face of the B-ring and the β face of the C-ring (Fig. 2a). The positions of these preformed cavities are similar to those predicted from binding studies¹².

This structure is the first example of an LBD from the steroid class of nuclear receptors, and provides an instructive model for members of this family. A similar overall hormone-binding mode is anticipated with the A-ring probably bound between H3 and H6 by an arginine (homologue of Arg 394) and a glutamine (homologue of Glu 353). This exclusive replacement of the Glu 353 of ER by a glutamine fulfils the hydrogen-bonding requirements of the 3-keto steroids. The model proposed for the ligand binding mode of dexamethasone in the human glucocorticoid receptor⁹, in which the D-ring binds between H3 and H6, should therefore be re-examined in the light of our observations.

RAL is a clinically relevant selective antagonist that specifically counters the mitogenic effects of E_2 in the reproductive tissues, while maintaining beneficial oestrogenic effects in other tissues^{5,14}.

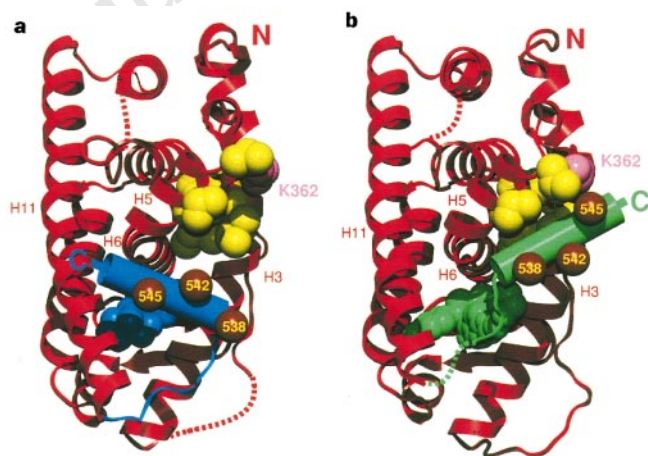


Figure 3 Positioning of helix H12. Position is shown in **a**, the ER LBD- E_2 complex; and **b**, the ER LBD-RAL complex. H12 is drawn as a cylinder and coloured blue (E_2 complex) or green (RAL complex). The remainder of the ER LBD is shown in red. Dotted lines indicate unmodelled regions of the structures. Hydrophobic residues located in the groove between H3 and H5 (yellow) and Lys 362 (K362, pink) are depicted in space-filling form. The locations of Asp 538, Glu 542 and Asp 545 are highlighted (brown spheres) along with the helices that interact with H12 in the two complexes.

RAL binds at the same site as E_2 within the LBD (Fig. 2b,d), with the hydroxyl group of the benzothiophene moiety (O3; see Fig. 2d for atom numbering) mimicking the A-ring phenolic hydroxyl of E_2 by binding in the polar pocket between H3 and H6. In contrast, the binding mode of RAL at the 'D-ring end' of the cavity, between H8 and H11, is markedly different from that of E_2 . Although the phenolic hydroxyl (O11) hydrogen bonds with His 524, it is displaced 5.1 Å from the position occupied by the 17 β -OH in the E_2 complex. Consequently, the imidazole ring of His 524 rotates in the RAL complex to compensate for the change in oxygen position and to maintain a favourable hydrogen-bonding position. The remainder of the core is involved in non-polar contacts similar to those seen for E_2 . The side chain of RAL makes extensive hydrophobic contacts with H3 and H5/6, H11 and the loop between H11 and H12. It is anchored to the protein by a direct hydrogen bond between Asp 351 and the piperazine ring nitrogen (N26). However, at over 11 Å in length, the side chain is too long to be contained within the confines of the binding cavity, and instead it displaces H12 and protrudes from the pocket between H3 and H11. This helix displacement is anticipated to be a general feature of both steroidal and non-steroidal anti-oestrogens that possess a bulky side-chain substituent. The importance of the narrow cleft at the A-ring end of the cavity in determining the overall ligand-binding mode is highlighted by the observation that RAL's benzothiophene moiety occupies the same spatial position as the A and B rings of E_2 . The alternate D-ring binding mode of RAL presumably arises as a result of both the inflexibility of the arylbenzothiophene core and the limited scope for positioning the side chain. The orientation of E_2 and RAL should allow the accurate positioning of most of ER's ligands, but further structural studies will be required to understand both the cavity's plasticity and the reported range of different binding modes¹⁵.

The LBD's transcriptional activation function (AF-2) can interact with a number of putative transcriptional coactivators in a ligand-dependent manner^{4,16-18}. Helix 12 is essential for such transactivation as both loss or mutation in this region results in a receptor that is unresponsive to ligand¹⁹. Mutational analyses in both ER and other nuclear receptors^{20,21} have identified several additional residues that influence the function of AF-2, suggesting that the LBD's coactivator recruitment surface, although centred on H12, probably also encompasses parts of the surrounding helices H3, H5/6 and H11.

In the E_2 -liganded complex, H12 sits snugly over the ligand-binding cavity and is packed against H3, H5/6 and H11. Although it makes no direct contact with E_2 , it forms the 'lid' of the binding cavity and projects its inner hydrophobic surface towards the bound hormone. Its charged surface, comprising Asp 538, Asp 545 and the highly conserved Glu 542, is directed away from the body of the LBD on the side of the molecule lying perpendicular to the dimerization interface (Fig. 3a). This precise positioning of H12, which is observed in all known structures of the liganded forms of the LBD^{6,7}, seems to be a prerequisite for transcriptional activation as, by sealing the ligand-binding cavity, it generates a competent AF-2 that is capable of interacting with coactivators. In contrast, the alignment of H12 over the cavity is prevented by RAL, and instead the helix lies in a groove formed by H5 and the carboxy-terminal end of H3. This antagonist-induced repositioning of H12 involves a rotation of 130° combined with a 10-Å rigid-body shift towards the amino terminus of the LBD compared with the agonist-induced conformation (Fig. 3b). The complementarity of this hydrophobic groove to the inner surface of H12 suggests that its positioning in the RAL complex represents a real conformation rather than an artefact produced by the crystal lattice. A highly conserved lysine residue (Lys 362), which is required for efficient E_2 -dependent recruitment of certain coactivators²¹, is located at one end of this hydrophobic groove, and is partly buried by the reoriented helix. Taken together, these observations provide compelling evidence

that the antagonistic properties of RAL are based on its ability to prevent the formation of a transcriptionally competent AF-2 conformation. The movement of H12 clearly disrupts the overall surface topography of AF-2, but it is feasible that the tissue selectivity of RAL may reside in its ability to occlude particular coactivator recruitment sites on the surface of the ER LBD.

Selective antagonism of the kind exhibited by RAL is a complicated phenomenon that arises through the interplay of a number of factors, such as differential ligand effects on the transactivation functionalities of the ER, the type of coactivator recruited, and the cell and promoter context^{3,4,22,23}. Nevertheless, our data on these structures give valuable insights into the binding of ligands to this receptor, and provide the basis for the structure-based design of improved agonists and antagonists for the treatment of oestrogen-related diseases. □

Methods

Protein purification and crystallization. The LBD of human ER- α (residues Ser 301 to Thr 553) was expressed, purified and carboxymethylated as described²⁴. ER LBD-E₂ and LBD-RAL complexes were prepared by including 75 μ M of the respective ligand in the column elution buffer. The ER LBD is particularly refractive to crystallization, and carboxymethylation of the free thiol groups was essential for growing crystals suitable for diffraction studies. Examination of the electron-density maps shows that Cys 381 is uniformly modified and the remaining three cysteines are either unmodified (Cys 447) or in flexible regions of the structure. The ER LBD-RAL and LBD-E₂ complexes were crystallized using the hanging-drop technique at 18 °C. For the RAL complex, the reservoir solution contained 12% (w/v) PEG 4000, 0.2 M magnesium chloride, 50 mM L-lysine, 0.1 M sucrose and 5% 1,4-dioxane in 0.1 M Tris-HCl, pH 8.5. Hanging drops were composed of equal volumes of protein (7.2 mg ml⁻¹) and reservoir solutions. Monoclinic crystals, belonging to the space group C2 with unit cell dimensions $a = 104.53$ Å, $b = 53.68$ Å, $c = 102.71$ Å, $\beta = 116.79^\circ$ and containing one ER LBD dimer per asymmetric unit, appeared within 2–4 weeks. Two other crystal forms were grown by subtle manipulation of the crystallization conditions (C2, $a = 89.91$ Å, $b = 75.09$ Å, $c = 87.50$ Å, $\beta = 103.01^\circ$; C222₁, $a = 65.47$ Å, $b = 95.99$ Å, $c = 168.14$ Å). For the E₂ complex, drops containing equal volumes of protein (7–13 mg ml⁻¹) and reservoir solution were equilibrated against 0.1 M Tris-HCl, pH 8.1, 2.4 M ammonium formate and 8% dimethylsulphoxide. The E₂ complex crystals belong to the space group P2₁, with unit cell dimensions $a = 61.48$ Å, $b = 115.16$ Å, $c = 137.38$ Å, $\beta = 98.8^\circ$, and contain three ER LBD dimers per asymmetric unit.

Data collection, phasing and refinement. For the ER LBD-RAL complex, native diffraction data were collected from a single frozen crystal (120 K) on beamline X11 at EMBL (DESY, Hamburg). Heavy-atom derivatives were collected in-house from flash-frozen crystals. Data were integrated and reduced using the programs DENZO and SCALEPACK²⁵. MIR analysis was performed using the CCP4 suite of programs²⁶. Diffraction data for the alternate C2 (York) and C222₁ (DESY, Hamburg) crystal forms were collected to resolutions of 3.0 and 3.1 Å, respectively. Initial phases were calculated to 3 Å using MLPHARE²⁶ and subsequent two-fold averaging, non-crystallographic matrix refinement and phase extension were carried out using DM²⁶. An initial polyalanine trace was used to generate a dimeric search model which was correctly positioned in the alternate C2 and C222₁ crystal forms using molecular replacement (AMoRe²⁶). Twenty cycles of cross-averaging between all three crystal forms were carried out with DMMULTI²⁶, using only the MIR phase information. The resultant electron-density map showed no bias towards the input model and enabled the unambiguous tracing of the remainder of the molecule and the assignment of most of the amino-acid sequence. Refinement was performed with REFMAC²⁷ using bulk solvent corrections and anisotropic scaling. All data between 25 and 2.6 Å were included with no sigma cut-offs. Tight non-crystallographic restraints were maintained during the initial cycles but were loosened in the final stages of refinement. Phases from multiscrystal averaging were included at all stages and individual atomic temperature factors were refined isotropically. The final model comprises residues 307–459, 470–528 and 535–547. The missing regions correspond to flexible loops between helices H9 and H10 (460–469) and H11 and H12 (529–534) and the chain termini.

Residues Tyr 331(A), Asp 332(A), His 377(B), Glu 397(AB), Lys 416(AB), Glu 419(AB), Glu 423(B), Leu 469(B), Glu 470(AB), Glu 471(AB), Lys 472(AB), Arg 477(AB), Lys 492(A), Glu 542(A), Arg 548(B) and Leu 549(B) were poorly resolved in the electron-density maps and not fully modelled.

For the ER LBD-E₂ complex, diffraction data were collected at room temperature from a single ER LBD-E₂ crystal on beamline X11 at EMBL (DESY, Hamburg). Initial phase estimates were obtained with AMoRe using the refined ER LBD-RAL dimer as a search model. The correct solution, corresponding to three ER LBD dimers, had a correlation coefficient of 69.8 and an *R*-factor of 40.6 after AMoRe rigid-body refinement. Six-fold averaging was performed using DM and the structure was refined with REFMAC using tight non-crystallographic restraints, averaged phases from DM, bulk solvent corrections and anisotropic scaling. All data between 20 and 3.1 Å were included with no sigma cut-offs. A single, overall *B*-value was applied in the early stages of refinement until the *R*_{free} converged. Subsequent cycles used tightly constrained, full isotropic *B*-value refinement. The final model for each monomer comprises residues 305–548 but includes two unmodelled loops between residues 331–336 and 462–464. The first four (301–304) and last five (549–553) residues are disordered. The side chains of Leu 306, Leu 466, Leu 469, Lys 492, Lys 531 and Leu 536 were poorly resolved in the electron-density maps and not modelled beyond their C β atoms. All model building was carried out using the graphics package QUANTA (Molecular Simulations Inc., San Diego).

Illustrations. Figures 1, 2a,b, 3 were prepared with QUANTA (Molecular Simulations Inc., San Diego); Fig. 2c, d was prepared with LIGPLOT²⁸.

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Correspondence and requests for materials should be addressed to R.E.H. (e-mail: rod@york.york.ac.uk). Coordinates have been deposited at the Brookhaven Protein Data Bank, accession codes 1ERE for the oestradiol-liganded structure and 1ERR for the raloxifene-liganded structure.

Structure at 1.65 Å of RhoA and its GTPase-activating protein in complex with a transition-state analogue

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Small G proteins of the Rho family, which includes Rho, Rac and Cdc42Hs, regulate phosphorylation pathways that control a range of biological functions including cytoskeleton formation and cell proliferation^{1–7}. They operate as molecular switches, cycling between the biologically active GTP-bound form and the inactive GDP-bound state. Their rate of hydrolysis of GTP to GDP by virtue of their intrinsic GTPase activity is slow, but can be accelerated by up to 10⁵-fold through interaction with rhoGAP, a GTPase-activating protein that stimulates Rho-family proteins^{8,9}. As such, rhoGAP plays a crucial role in regulating Rho-mediated signalling pathways. Here we report the crystal structure of RhoA and rhoGAP complexed with the transition-state analogue GDP.AIF₄[−] at 1.65 Å resolution. There is a rotation of 20 degrees between the Rho and rhoGAP proteins in this complex when compared with the ground-state complex Cdc42Hs.GMPPNP/rhoGAP, in which Cdc42Hs is bound to the non-hydrolysable GTP analogue GMPPNP¹⁰. Consequently, in the transition state complex but not in the ground state, the rhoGAP domain contributes a residue, Arg 85_{GAP} directly into the active site of the G protein. We propose that this residue acts to stabilize the transition state of the GTPase reaction. RhoGAP also appears to function by stabilizing several regions of RhoA that are important in signalling the hydrolysis of GTP.

It has been proposed that GAPs stimulate the intrinsic GTPase activity of the Ras superfamily of proteins either by contributing residues that participate directly in catalysis or by performing an allosteric function (reviewed in ref. 11). We have investigated this question by first determining the structure of p50rhoGAP alone¹² and then as a complex with Cdc42Hs.GMPPNP¹⁰. From the former structure, we were able to identify residues that could be involved in G-protein binding and GTPase activation. Comparison of this rhoGAP structure with that of p120rasGAP reveals that, although both molecules are predominantly α-helical, there is no tertiary structural similarity, which is consistent with the lack of any detectable sequence homology between them^{10,13}. From the

Table 1 Data collection and refinement statistics

Crystal space group: $P2_12_12_1$	
Cell parameters (Å): $a = 66.5, b = 72.0, c = 91.3$	
Data processing	
Observations to 1.65 Å	160,124
Unique reflections	42,992
Completeness (%)	80.5 (60.8)
I/σ	16.1 (2.3)
R_{merge} (%) [*]	6.5 (32.4)
Refinement	
Data range (Å)	6.0–1.65
Reflections ($F > 0$)	37,875
Non-hydrogen atoms	2,991
Solvent molecules	497
R.m.s. Δ bond length (Å) [†]	0.013
R.m.s. Δ bond angles (deg) [†]	1.6
R.m.s. ΔB-factors for bonded atoms [‡]	2.0
R_{free} (%) [§]	21.5
R_{cryst} (%) [§]	16.9

Values in parentheses correspond to the highest-resolution shell (1.76–1.65 Å)

^{*} $R_{\text{merge}} = \sum_i |I_i - \langle I \rangle| / \sum_i I_i$, where I_i is the observed intensity and $\langle I \rangle$ is the average intensity.

[†]Root-mean-squared deviation (R.m.s. Δ) are given from ideal values.

[‡] R_{free} is the same as R_{cryst} , but calculated on the 5% of data excluded from refinement.

[§] $R_{\text{cryst}} = \sum_i |F_o - F_c| / \sum_i F_o$ for all reflections, where F_o and F_c are the observed and calculated structure amplitudes respectively.

Cdc42Hs.GMPPNP/rhoGAP crystal structure, we were able to show how these two proteins interact in the presence of GMPPNP¹⁰. Although no interaction between the invariant Arg 85_{GAP} and the phosphate moiety of the nucleotide was evident in that complex, we concluded that such an interaction could occur during GTP hydrolysis. We anticipated that Arg 85_{GAP} would contribute to catalysis by stabilizing the transfer of charge during transition-state formation, an idea supported by data obtained from the transition-state analogue GDP.AIF₄[−]: binding of p50rhoGAP to RhoA.GDP is enhanced 100-fold in the presence of AIF₄[−], and this increased binding is abolished if Arg 85_{GAP} is mutated¹⁰. These results suggest that AIF₄[−] acts in the presence of p50rhoGAP and RhoA.GDP in a manner similar to that seen in other phospho-transferring enzymes. The crystal structures of several such enzymes, including the G protein G_{iα1} (ref. 14), transducin¹⁵ and myosin S1 (ref. 16), have been analysed as complexes with nucleoside diphosphate and AIF₄[−]. These all reveal that the aluminium ion is octahedrally coordinated (with four fluorine atoms defining the equatorial plane), located at a site broadly equivalent to the γ-phosphate position, and coordinated at the apical positions by a β-phosphate oxygen on one side and a water molecule on the other. This arrangement cannot exactly represent the trigonal bipyramidal transition state of a phosphoryl-transfer reaction, although it is probably a good approximation. To examine the proposed role of Arg 85_{GAP} in transition-state stabilization, we have crystallized and determined the structure of p50rhoGAP and RhoA.GDP in the presence of AIF₄[−] (Table 1).

The overall structure of the RhoA.GDP.AIF₄[−]/rhoGAP complex is shown in a ribbons representation in Fig. 1a. Alignment of the G-protein components of the ground- and transition-state complexes reveals a substantial rearrangement between the G protein and its GAP (Fig. 1b). The two complexes contain different G-protein homologues because we could not crystallize p50rhoGAP with Cdc42Hs.GDP in the presence of AIF₄[−]. However, the residues that contribute to the interface with GAP are strictly conserved between Cdc42Hs and RhoA, suggesting that it is the presence of the transition-state analogue that causes rearrangement of the heterodimer. The movement is essentially a 20° rigid-body rotation of rhoGAP about an axis that runs close to the phenolic hydroxyl of Tyr 66_{Rho}. There is a small local change in the structure of the A–A1 loop of GAP which, in combination with the rigid-body rotation, enables the invariant Arg 85_{GAP} to interact with the nucleotide. Indeed, the movement of Arg 85_{GAP} during the catalytic cycle appears to be the key to the function of p50rhoGAP.

In the transition-state complex, the heterodimer interface buries $2,280 \text{ \AA}^2$ of solvent-accessible surface and comprises an extensive network of both direct and water-mediated hydrogen bonds (Fig. 2 and Table 2). The binding surface on rhoGAP involves the A–A1 loop and the B and F helices. These three elements are responsible for most of the interactions with the G protein and define a shallow pocket on rhoGAP lined largely with conserved residues¹². The switch II, effector loop and P-loop regions of RhoA fit into this conserved pocket and account for a substantial proportion of the

interface. In particular, the switch II region seems to act as a pivot point around which rigid-body motion occurs on progression from the ground state to the transition state. In the transition-state but not the ground-state complex, hydrogen-bonding interactions occur between α -helix H3 of RhoA and the C-terminal end of the A–A1 loop of rhoGAP. These α -H3_{Rho}-mediated interactions account for most of the $460 \text{ \AA}^2$ increase in buried surface area observed in the transition-state complex structure.

Initial electron-density maps around the active-site region clearly

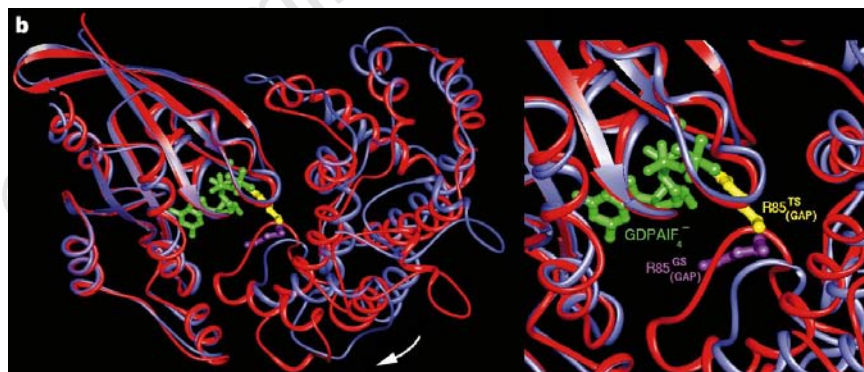
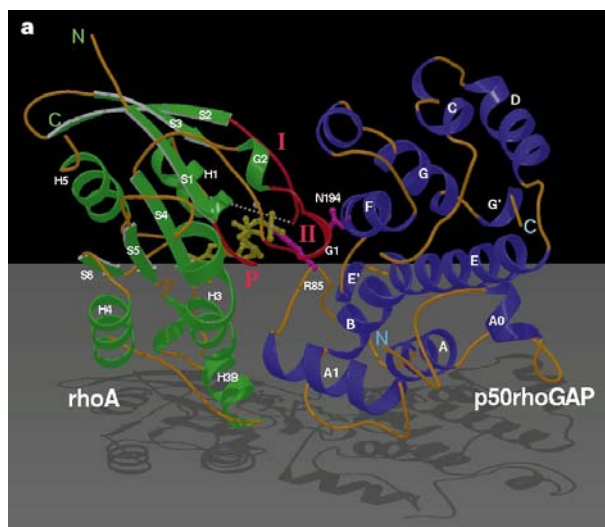


Figure 1 a, Overall view of the complex between RhoA.GDP.AIF₄[−]/p50rhoGAP viewed along the heterodimer interface. The G protein is coloured green, the bound GDP.AIF₄[−] is shown in yellow, and the GAP molecule is blue. The switch I, II and P-loop regions of RhoA are highlighted in red, and two residues, Arg 85 and Asn 194, from rhoGAP are highlighted in magenta³⁰. Residues 31–33 of RhoA are disordered and the resulting break in the structure is shown by the dotted line. **b**, Comparison of the relative orientations of rhoGAP in the ground state

Cdc42Hs.GMPPNP complex (blue) and the transition state RhoA.GDP.AIF₄[−] complex (red) following least-squares superposition of the G proteins (r.m.s. = $0.9 \text{ \AA}$ for overlap of 170 C α atoms). The arrow indicates a 20° rotation of the GAP component in moving from the ground state to the transition state. The side-chain positions of Arg 85 in the ground state and transition state are shown in magenta and yellow, respectively²⁹, and the nucleotide is shown in green.

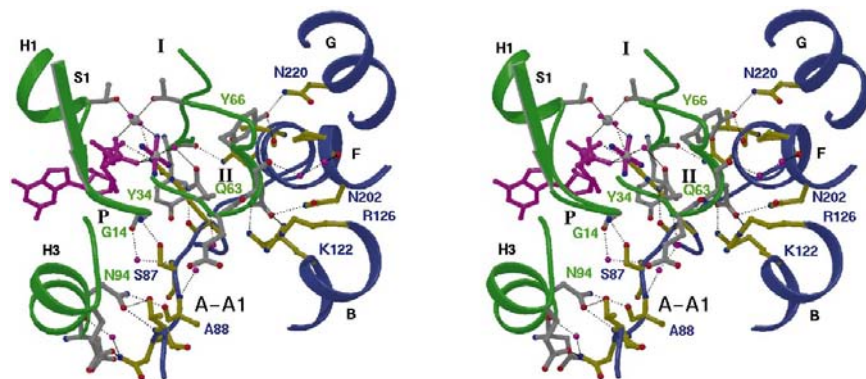


Figure 2 Stereo ball-and-stick representation of the complete RhoA.GDP.AIF₄[−]/p50rhoGAP interface viewed in an orientation similar to that in Fig. 1a. The carbon atoms of RhoA are shown in grey and those of rhoGAP are in yellow. GDP.AIF₄[−] is coloured magenta and water molecules are shown as small magenta spheres.

showed the structure of GDP.Mg and AlF_4^- before coordinates for these groups were included in the atomic model. The refined structure shows the AlF_4^- to be almost exactly square-planar, with the aluminium ion 1.92 Å from the β -oxygen of the GDP and 1.87 Å from the hydrolytic water molecule (Fig. 3a,b). Two important points arise from these data. First, the aluminium species that mimics the transition state for this system is AlF_4^- and not AlF_3 , which has been observed in certain other phosphotransferring enzymes^{17,18}. In the crystallization mixture, both aluminium species will be present, albeit with an excess of AlF_4^- . In the case of RhoA/rhoGAP, it appears that AlF_4^- has a higher affinity for the active site. Second, inasmuch as $\text{GDP}.\text{AlF}_4^-$ mimics the transition state of the reaction, the bond lengths between the apical oxygen ligand and the aluminium imply that the hydrolytic reaction is associative.

From this structure, three residues appear to be particularly important in GAP-activated GTP hydrolysis. These are Gln 63_{Rho}, Arg 85_{GAP} and Asn 194_{GAP}. Gln 63 of RhoA is structurally and functionally equivalent to Gln 61 of Ras, Gln 61 of Rac and Gln 204 of G_{12i1}, all of which have been proposed to participate in GTP hydrolysis¹⁹. Gln 63 in this complex is substantially better ordered than its equivalents in Ras²⁰, Rac²¹ or Cdc42Hs.GMPPNP/p50rhoGAP¹⁰. Indeed, the whole of the switch II region is better ordered in this complex because of its interactions with rhoGAP. In particular, Asp 65_{Rho} and Tyr 66_{Rho} are involved in an extensive hydrogen-bonding network with Lys 122_{GAP}, Arg 126_{GAP}, Val 197_{GAP}, Asn 202_{GAP} and Asn 220_{GAP}. The rigid-body movement on going from the ground state to the transition state also means that the side chain of Gln 63_{Rho} is stabilized through interaction with the main-chain carbonyl of Arg 85_{GAP} (Fig. 3b). Consequently, only in this transition-state complex does Gln 63_{Rho} hydrogen-bond to a water molecule that is directly in line with the β -oxygen of GDP and the

aluminium ion. We suggest, therefore, that the role of Gln 63_{Rho} in rhoGAP-activated GTP hydrolysis is to position the hydrolytic water molecule. The stabilization of the Gln 63_{Rho} side chain by the main-chain carbonyl of Arg 85_{GAP} concomitantly restricts the freedom of the hydrolytic water molecule and thus reduces the entropy barrier to transition-state formation. Deamidation of Gln 63_{Rho} by *Escherichia coli* cytotoxic necrotizing factor-1 is the means by which these toxins cause tissue damage and death of the host^{22,23}. Similarly, the Q63E_{Rho} mutation abolishes the intrinsic GTPase activity and is no longer stimulated by rhoGAP. These data appear to disagree with results from a similar mutation in Ras¹⁹, but they are consistent with the central role of Gln 63_{Rho} in the GAP-enhanced GTP hydrolysis suggested by our transition-state complex structure.

We have argued previously on the basis of both structural and sequence conservation that, in addition to Arg 85_{GAP}, Asn 194_{GAP} could also be important in rhoGAP function¹². Asn 194_{GAP} is the only polar amino-acid that is conserved among all rhoGAP domains that activate GTP hydrolysis. In the RhoA.GDP. AlF_4^- /p50rhoGAP complex, the side chain of Asn 194_{GAP} interacts with the main-chain carbonyl of Tyr 34_{Rho}, apparently stabilizing the effector loop. This loop is disordered in the structure of Rac1.GMPPNP²¹. In this heterodimeric structure, Tyr 34_{Rho} and subsequent effector loop residues are well ordered and have thermal *B*-factors lower than the average for the rest of the complex. It is interesting therefore that mutation of Asn 194_{GAP} to a valine produces a mutant rhoGAP that stimulates RhoA-mediated GTP hydrolysis by 2×10^3 (50-fold less than wild type; J.F.E., unpublished results). The means by which effector-loop stabilization contributes to increased GTP hydrolysis is probably mediated by interaction of Thr 37_{Rho} with the nucleotide-associated Mg^{2+} ion. The six groups acting as ligands to the

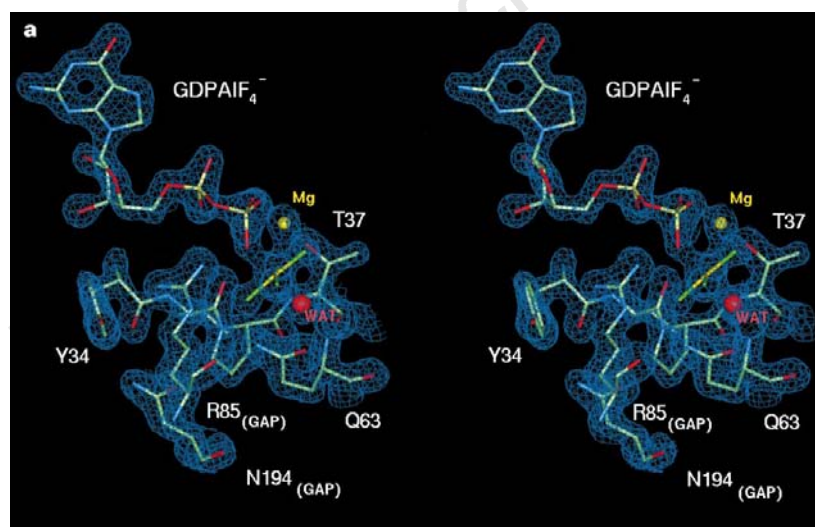
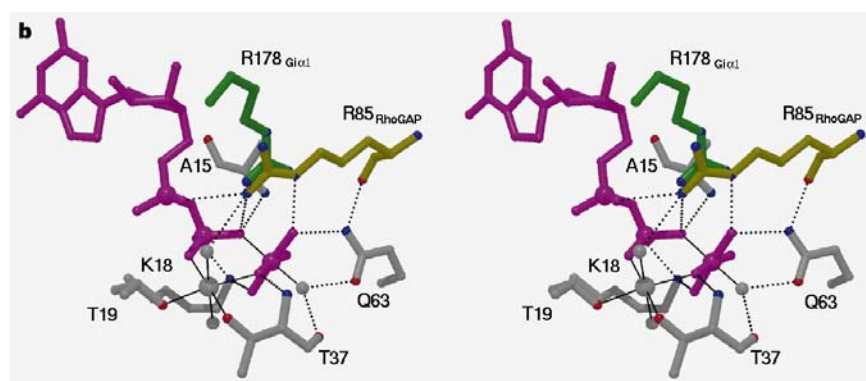


Figure 3 a, Stereo view of a portion of the final $2F_o - F_c$ electron density map around the nucleotide-binding site on RhoA contoured at 2.0σ , with the final refined atomic model for selected residues from RhoA and GAP superimposed. Carbon, white; oxygen, red; nitrogen, blue; fluorine, green. The proposed hydrolytic water molecule is shown in red. **b**, Stereo ball-and-stick representation of the interactions around the diphosphate/ AlF_4^- of the nucleotide. Residues from rhoGAP are coloured yellow with those of rhoA in grey. The position of Arg 178_{G12i1} following overlap of the G-protein component of G_{12i1} with RhoA is shown in green.



octahedrally coordinated Mg^{2+} ion are all well ordered in this complex, with *B*-factors that are all lower than the average *B*-factor of the complex. Stabilization of the effector loop at Tyr 34_{Rho} therefore seems to reduce the mobility of Thr 37_{Rho}, which in turn orders and positions the Mg^{2+} and γ -phosphate optimally for hydrolytic attack.

The transition-state complex described here shows how Arg 85_{GAP} interacts with the β - γ bridging oxygen and one of the fluorides of the AlF_4^- (Fig. 3b). The positioning of the guanidinium group would enable it to stabilize developing negative charge on either the β - γ bridging oxygen or the equatorial oxygens of the trigonal bipyramidal phosphoryl group. There is still controversy as to whether GAP-enhanced GTP hydrolysis by small G proteins proceeds by an associative or dissociative mechanism, or by some mixture of both²⁴. In the case of an associative mechanism, negative charge will develop on the equatorial oxygens of the trigonal bipyramidal transition state; in a dissociative mechanism, the β - γ bridging oxygen will experience the largest increase in negative charge. The positioning of the guanidinium group of Arg 85_{GAP} and the distribution of charge within this group means that both mechanisms could be enhanced by the interaction of the side chain of Arg 85_{GAP} with the transition state.

The function of Arg 85_{GAP} appears to be closely related to that of Arg 178 of $G_{i\alpha 1}$. These two arginine residues are shown in Fig. 3b after the Ras-like domain of $G_{i\alpha 1}$ has been least-squares fitted against the Rho component of this complex. The C α positions of the two residues are 10 Å apart, reflecting the fact that Arg 178 _{$G_{i\alpha 1}$} is located on an extension of the effector loop of its Ras-like domain which has no structural counterpart in p50rhoGAP. Despite this, the carbon atoms of the two guanidinium groups superpose with an r.m.s. of 0.5 Å, and the guanidinium group of Arg 85_{GAP} as a whole, overlaps in a pseudosymmetric fashion with Arg 178 _{$G_{i\alpha 1}$} with an r.m.s. of 0.7 Å. The fact that these two disparate systems utilize arginine residues in such similar ways indicates that the stereochemical requirements of phosphoryl transfer are probably exacting. The structure of the Ras.GDP. AlF_3 /rasGAP complex at 2.5 Å resolution¹³ shows that the guanidinium group of the invariant Arg 789_{rasGAP} also interacts with the phosphate/aluminium fluoride moiety of the nucleotide. In addition to the well documented similarities between the GTP-binding sites of Ras and Rho family proteins, there is a close correspondence between the interactions made by the carbonyl groups of Arg 789_{rasGAP} and Thr 35_{RAS}, the side chain of Gln 61_{RAS} and the hydrolytic water molecule and those observed in our complex (Arg 85_{GAP}, Gln 63_{Rho}, Thr 37_{Rho} and the attacking water; Fig. 3b). The similarity of these interactions is all the more remarkable given the lack of tertiary structural homology between rhoGAP and rasGAP. In particular, there are two important differences between the Rho/rhoGAP and Ras/rasGAP transition state complexes at their active sites. First, the Arg 789_{rasGAP} side-chain conformation does not seem to result in the rather precise overlap of the guanidinium groups seen in Rho/rhoGAP, $G_{i\alpha 1}$ and transducin (Fig. 3b)^{14,15}. Second, the aluminium ion in the Ras/rasGAP structure appears to be coordinated by three fluorine atoms and not four as observed in our complex structure, that of $G_{i\alpha 1}$ /RGS4 (ref. 25) and the $G_{i\alpha 1}$ (ref. 14)/transducin¹⁵ transition-state analogue crystal structures reported previously. Although these discrepancies may reflect real differences in the detailed stereochemistry of catalysis in the Ras/rasGAP system, a more definitive comparison awaits X-ray analysis of the Ras/rasGAP complex at high resolution.

The rhoGAP mutation R85K indicates that the enhancement of GTPase activity by rhoGAP is only partially accounted for by transition-state stabilization by Arg 85_{GAP}. From the structure and analysis presented here, it is clear that additional GAP enhancement is derived from stabilization of the switch regions of RhoA. This activity is analogous to the function ascribed to RGS4, a $G_{i\alpha 1}$ GAP, on the basis of the crystal structure of the $G_{i\alpha 1}$ /RGS4 complex²⁶ and also seems to be the case in the Ras/rasGAP complex¹³.

Table 2 Hydrogen-bonding interactions between RhoA.GDP. AlF_4^- and rhoGAP

rhoGAP			RhoA	
Residue	Atom	Distance (Å)	Atom	Residue
Arg 85	O	2.9	NE2	Gln 63
	NE	2.7	F3	GDP. AlF_4^-
	NH ₂	2.9	O2A	GDP. AlF_4^-
		3.1	O2B	GDP. AlF_4^-
Ser 87	OG	2.8	N	Gly 14
Ala 88	O	3.1	ND2	Asn 94
Asn 89	ND2	3.1	OE2	Glu 97
Thr 90	N	3.0	OD1	Asn 94
	OG1	3.0	OD1	Asn 94
Lys 122	NZ	2.7	OD2	Asp 65
Arg 126	NH1	2.8	OD1	Asp 65
Asn 194	ND2	2.8	O	Tyr 34
Val 197	O	2.6	OH	Tyr 66
Asn 220	ND2	3.0	OH	Tyr 66
Asn 202	ND2	2.8	OD1	Asp 65

The conformational rearrangement that occurs in progression from the ground state (GMPPNP) to the transition state (GDP. AlF_4^-) appears to be crucial for GTPase activation. There are two other G-protein complexes for which ground- and transition-state structures are available. In both $G_{i\alpha 1}$ and transducin, positioning of the catalytic arginine side chains does not require a significant conformational change because in these cases the arginines are not supplied by a separate GAP molecule. A key effect of the rotational rearrangement observed in our transition-state complex is to position Arg 85_{GAP} correctly with respect to the nucleotide. The interactions of the arginine guanidinium with the diphosphate/ AlF_4^- and its main-chain carbonyl with the side chain of Gln 63_{Rho} are only made possible by the large rearrangement and both are critical for GAP function. These interactions cannot occur in the ground-state structure which uses a markedly different and less extensive protein-protein interface. Initially, the G protein/GTP binds to rhoGAP to form the ground-state complex. The relative rotation between the G protein and rhoGAP then introduces Arg 85_{GAP} into the active site to neutralize the evanescent charge and activate the reaction.

This mechanism is not dissimilar to that proposed²⁷ for phosphoglycerate kinase. This enzyme catalyses phosphoryl transfer from 1,3-bisphosphoglycerate to ADP. The two substrates bind to separate domains of a bilobal enzyme and are substantially separated in the open conformation. Each substrate appears to induce small, local conformational changes which act synergistically to produce a 32° hinge-bending motion of one lobe with respect to the other. This movement simultaneously brings the two substrates together and, importantly, brings the conserved catalytic Arg 39 into the active site. This arginine is located on the opposite lobe to that which binds the nucleotide. In the open conformation, it is located ~10 Å from its final position in the ternary complex. Thus, in both Rho/rhoGAP and phosphoglycerate kinase, phosphoryl transfer is facilitated by substantial inter- or intramolecular rearrangements. These movements bring a catalytic arginine, initially distant from the nucleotide, into the active site to stabilize the transition state. □

Methods

Crystallization. RhoA was expressed as a GST-fusion protein using the pGEX-2T vector. After cleavage of the fusion protein with thrombin there are three extra, GST-derived residues at the N terminus but with the first methionine of RhoA missing. The RhoA protein also carries a point mutation (F25N) to improve its stability. Human p50rhoGAP (fragment 198–439) was expressed and purified as described¹². Crystals were grown in hanging drops by vapour diffusion using equal amounts of reservoir solution (containing 18% PEG2000 MME (Sigma), 100 mM MES, pH 6.0, 10 mM $MgCl_2$, 3 mM DTT, 3 mM $NaNa_3$, 114 mM $(NH_4)_2SO_4$) and stock solution of the protein complex at 400 μ M in

20 mM Tris.HCl, pH 7.4, with 1 mM AlCl₃ and 10 mM NaF. Crystals belonged to space group $P2_12_12_1$ ($a = 66.5 \text{ \AA}$, $b = 72.0 \text{ \AA}$, $c = 91.3 \text{ \AA}$, which with one complex per asymmetric unit contains 45% solvent) and although they were of modest size ($200 \times 20 \times 30 \mu\text{m}^3$), they were very well ordered and diffracted to high resolution.

Data collection and processing. Diffraction datasets were collected from single crystals at 100 K using an Oxford Cryosystems device. Crystals were prepared for flash cooling by immersion in mother liquor made up with 20% PEG400. Two native data sets were collected, the first to 2.1 Å at Daresbury 9.6, the second at 1.65 Å at Daresbury 7.2. Both datasets were collected using a large MAR image plate detector and data were integrated and scaled using DENZO and SCALEPACK. All subsequent calculations were carried out with the CCP4 program suite²⁸.

Structure determination and refinement. The structure of the complex was solved by molecular replacement (AMORE²⁹). The crystallographic models used were the two separate components of the Cdc42Hs.GMPPNP.p50rhoGAP complex (Brookhaven accession code 1AM4). Although there is sequence variation between the two Rho family members Cdc42Hs and RhoA, the molecular replacement solution was unambiguous, treating the two components of the complex separately. However, the molecular replacement did not work if the intact Cdc42Hs.GMPPNP.p50rhoGAP was used because of the rearrangement between the two proteins. After rigid-body refinement, the model was subjected to alternating rounds of reciprocal space refinement with REFMAC²⁸ and automated water building with ARP²⁸; the resulting electron density at 1.65 Å was of excellent quality. The Rho model was then rebuilt with the correct sequence using the graphics program O and then re-refined. At this stage ($R_{\text{cryst}} = 0.22$, $R_{\text{free}} = 0.25$) the electron density for the AlF₄⁻, GDP.Mg²⁺ and associated water molecules was clear and these moieties were included in the atomic model. After a further round of manual rebuilding, the final model was refined with good stereochemistry to $R_{\text{cryst}} = 16.9\%$ and $R_{\text{free}} = 21.5\%$; the average thermal B -factor for the protein was $24.0 \text{ \AA}^2$ (the Wilson B -factor was $21.5 \text{ \AA}^2$). Residues are numbered relative to the fragment used so that residue 1 of the rhoGAP fragment described here corresponds to residue 198 of the full-length p50rhoGAP protein.

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Correspondence and requests for materials should be addressed to S.J.S. (e-mail: s-smerdo@anika.nimr.mrc.ac.uk). Coordinates have been deposited with the Brookhaven Data Bank, accession code 1TX4.

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German fare leads off for Biotechnica

The Hannover Fairgrounds in Germany host the Biotechnica trade fair on 21–23 October. In 1995 there were 436 exhibitors from 17 countries, serving the biotechnology market. Here is some of what will be on view this year.

Pipet Helper

From Eppendorf

Assists in dispensing duties that use graduated glass pipettes

The user friendly design has no pipetting ball and provides one-handed, fatigue-free dispensing. This economical and safe alternative has been developed for all measuring pipettes and volumetric pipettes in the volume range from 0.1 to 100 ml. The speed for aspirating and dispensing can be controlled smoothly and precisely using the pipetting lever. Blow-out can be executed by pressing the minibellows with finger control. Moreover, it has a high aspirating speed of 25 ml in 5 s, and weighs in at a mere 125 g. Wet pipettes remain firmly in position with Pipet Helper and a hydrophobic membrane filter ensures that no liquid can enter the device.

Reader Enquiry No. 100

Pump systems

From Heidolph

An expanded and renewed pump-line

This product range now includes peristaltic pumps that have six pump drives with four pump heads. There are 36 choices of calibrated tubing with a range of flow rates from 0.16 to 6,068 ml min⁻¹, depending on the combination. Gear pumps are also offered, which utilise three gear-pump drives and three gear-pump heads to achieve flow rates of up to 3.5 l min⁻¹ (maximum pressure is 7 barr). Other pumps include centrifugal pumps. Six models are offered that can operate at flow rates of up to 75 l min⁻¹. There are also immersion pumps, of which there are three models for flow rates up to 20 l min⁻¹. Heidolph also offers OEM pumps that made according to a customer's requirements.

Reader Enquiry No. 101



Six pack: Heidolph pumps boast six drives and four heads.

Laser microscope system

From P.A.L.M.

Laser microbeam microdissection and pressure catapulting for 'non-contact' tissue, single-cell and chromosome micropreparation

The Robot-Microbeam is a tool for easy and quick microdissection and micropreparation of minute tissue areas, single cells or chromosomes. The focused 337-nm nitrogen laser yields a laser spot of less than 1 µm in diameter. The very high photon density within the laser focus is used to destroy any unwanted material, using non-thermal photodecomposition. Targets are brought into the line of the laser using the motorized, computer-controlled Robot-Stage, which is designed to operate with nanometre precision. The laser microbeam cuts a small, material-free gap around the specimen, accommodating irregular shapes and separating them from their surroundings. Laser pressure catapulting (LPC) directs samples into a tube with the photonic force of the slightly defocused microbeam.

Reader Enquiry No. 102

akku-drive

From Hirshman Laborgeräte

A battery-operated motor piston system that enables fully electronic dosage and titrating

The system consists of a basic unit that is screwed on the reagent bottle upon which exchangeable dispenser and titrator modules are mounted. These have integrated control electronics, keyboard and LC display. Simply changing between the modules changes the function. A Quick-Cal function enables the user to calibrate for special liquids, such as a highly viscous medium.

Reader Enquiry No. 103

BBD 6220 incubator

From Heraeus

A CO₂ incubator that offers in situ sterilization

This 220-l capacity CO₂ incubator has a built-in 180 °C, hot-air sterilization cycle, intended to destroy all viruses, bacteria and fungi commonly encountered in cell-culture applications. The disinfection process can be carried out with sensors and internal fittings in position, avoiding one source of contamination. Additional protection is provided by a unique pyrolytic germ barrier, which sterilizes the water vapour used for humidification before it reaches the working chamber. There is no water storage within the incubation chamber — water is stored externally in a readily accessible reservoir. The BBD 6220

controls the temperature, CO₂ level and relative humidity, and is equipped with a hot-air, jacketed heating system, which is said to prevent condensation from forming on the stainless steel interior, even at a relative humidity of 95 per cent. Over-temperature protection and an error diagnosis system supplement the safety features of the incubator. Moreover, after a power failure, any pre-set incubation settings are restored automatically. All operating parameters can be monitored, controlled and documented via an RS232 digital interface.

Reader Enquiry No. 104

Secondary standards

From Hellma

Secondary spectrophotometric calibration standards in the ultraviolet range

This set comprises various glass and liquid filters, and allows the user to check for stray radiation, spectral resolution, absorbance and wavelength accuracy of a spectrophotometer between 200 and 400 nm. The filters are calibrated individually and supplied with a certificate.

Reader Enquiry No. 105

Excelon filter capsules

From Schleicher & Schuell

Ready-to-use, pre-sterilized, devices for particle removal and sterile filtration of gases and fluids in the pH range 1–14

The devices are available in PES, PTFE and polypropylene membranes: PES capsules have highly asymmetric polyethersulphone membranes for the filtration of biological fluids; whereas, Excelon TE uses PTFE membranes and is recommended for sterile air venting applications and the filtration of organic solutions. The PP capsule is for pre-filtration techniques and for use with PES capsules. These capsule filters are designed to be a cost-effective means of scale-up from volumes of 1-l to 1,000 l with minimal loss of product.

Reader Enquiry No. 106

TLC thermal blotter

From Atto

For use in eastern blotting for micro-analysis of glycolipids and phospholipids

Two new methods have been described by Dr Takao Taki's group, Tokyo Medical and Dental University School of Medicine, Tokyo, using polyvinylidene difluoride (PVDF) membranes to detect enzymes involved in glycosphingolipid

metabolisms: one transferred from an HPTLC plate by thin-layer chromatography (TLC) blotting, and the other transferred from PAGED enzymes after being impregnated with sialylparagloboside (IV3 Neu Ac α -nLc₄Cer) as the enzyme substrate. This method is said to be useful for detecting subtle changes in glycosphingolipid expression on the cell surface and its biological function. Measuring 220 mm $\times$ 250 mm $\times$ 350 mm, (W $\times$ D $\times$ H) the TLC thermal blotter, developed by Atto, is capable of transferring glycolipids or phospholipids from a TLC plate to a PVDF membrane and does this in about a minute.

Reader Enquiry No. 107

Cell systems

TC-202A

From Medical Systems

A bipolar/monopolar temperature controller

This unit has a wide range of uses, including biological applications, wherein cells or tissues are heated or cooled when placed in one of several micro-incubators offered by the company. These include the open perfusion micro-incubator (PDMI-2), a patch slice micro-incubator (PSMI), a Leiden static micro-incubator (LU-CBI), a Leiden closed perfusion micro-incubator (LU-CPC-CEH) and a brain/tissue slice system (BSC-BU). Long-term experimentation or observation of temperature-sensitive cells can be carried out on the stage of a microscope with the TC-202A using the supplied thermistor. This option allows the desired chamber temperature to be dialed directly on the control. On the PDMI-2 and PSMI models, the temperature can be controlled with the internal thermistor, as it is attached to the heat-exchange plate of the

Peltier devices. The incoming perfusant is heated or cooled by this plate, preventing overheating or overcooling of the chamber should the chamber thermistor become uncovered. The device is designed for low electrical noise, even allowing noise-sensitive patch clamp recordings to be carried out. The actual measured temperatures (0–50 °C) are displayed digitally as the controller directs current to heat or cool rapidly to within 0.2 °C of the set point.

Reader Enquiry No. 108

175 cm² Flasks

From Corning Costar

Flasks for cell culture

These flasks are manufactured from optically clear virgin polystyrene and are treated for optimal cell attachment, says the manufacturer. Other features include individual printed lot numbers to aid in product traceability and a choice of cap styles that includes the original plug-seal, phenolic-style or vent-style caps.

Reader Enquiry No. 109

Cell disruption by homogenization

From MA-APV Homogenizer Group

The company has produced a 20-page booklet detailing the evolution of cell disruption techniques, including the strengths and limitations of each different method

Available free upon request, the book recounts the history of cell disruption from early attempts to release vitamins from yeast to the use of chemicals, enzymes, pressure, hand grinders, ball mills and ultrasound, among other methods. The booklet includes a large number of citations on high-pressure homogenization (15,000 p.s.i. and above), which, according to the manufacturer, is the most effective technology in full-scale production. APV Homogenizer Group's role in high-pressure cell disruption is noted, including the company's work in developing the Gaulin CD valve and Rannie equipment. (Full reference information is supplied.)

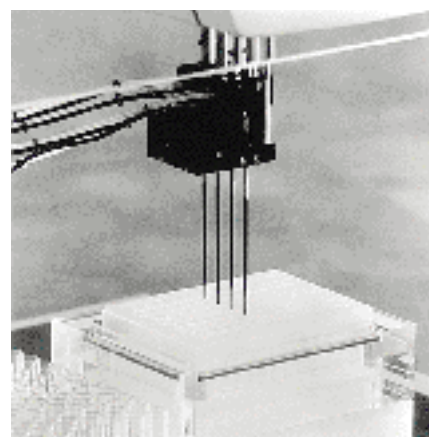
Reader Enquiry No. 110

IASys cuvettes

From Affinity Sensors

A new hydrophobic sensor surface for studying interaction analysis

This sensor surface allows hydrophobic binding of biomolecules, such as lipid monolayers and proteins. The surface should assist with studies of cell–receptor binding and provides an effective approach to understanding the influence of cell membranes on receptor–ligate interaction. The surface facilitates the formation of stable lipid monolayers from a



Help for the hydrophobic: a new sensor surface simplifies hydrophobic binding studies.

solution of lipid, replacing time-consuming liposome preparation to allow for kinetic and equilibrium measurements of ligate interaction. Lipid monolayers, formulated to resemble natural membranes, are used to form receptor–membrane complexes with specific receptor molecules. The interaction with specific ligate is observed using IASys optical biosensor technology. Purified receptor molecules are incorporated into the monolayer with their specific ligate binding activity intact. The IASys hydrophobic cuvette surface provides a complementary approach to that of the biotin surface. The streptavidin-coated biotin surface binds biotinylated membrane fragments or liposomes (lipid bilayers) in which transmembrane proteins can be assembled.

Reader Enquiry No. 111

EnVision

From Dako

A polymer-based staining technique gives heightened clarity

This visualization system has been formulated for use in immunohistochemistry and uses an enzyme-based, water-soluble polymer to which secondary antibodies are conjugated. Claiming increased assay sensitivity with just two incubation steps rather than the usual three, the system is said to be the first two-step method to achieve a sensitivity superior to streptavidin–biotin systems and similar methods. To make the system as easy to use as possible, it was designed with a short protocol and 10-min incubation steps. Use of this protocol, together with Dako's N-series ready-to-use primary antibodies, results in clear, reliable stainings within 40 min. EnVision+ was developed for situations where higher sensitivity is required. In this system, the user defines the primary antibody concentration and each incubation requires 30 min with a total assay time of 90 min. The major improvement made possible by this technol-

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ogy is that a large number of enzyme molecules (for example, horseradish peroxidase or alkaline phosphatase) can be bound to a dextran backbone together with specific binding reagents, such as secondary antibody molecules. The result is a high molecular mass polymeric conjugate consisting of up to 100 enzyme molecules and up to 20 antibody molecules per backbone.

Reader Enquiry No. 112

Mini-BeadBeater

From Biospec

A cell disrupter for small samples

The Mini-BeadBeater violently agitates a sealed microcentrifuge tube containing cells or tissue and hundreds of minute glass beads. Even resistant yeast or fibrous tissues are completely homogenized in 1–3 min in 0.1–1.0 ml of homogenization medium, says Biospec. The non-foaming, non-aerosol method preserves enzymes, nucleic acids and organelles, leaving products ready for PAGE, polymerase applications and diagnosis using antibody or oligonucleotide probes. It recovers intact RNA by simultaneous disruption and extraction in phenol or Gu-salt solutions. Sterile techniques are easily accommodated to recover intracellular virus and bacteria from plant and animal cells.

Reader Enquiry No. 113

Cytostar-T Assays

From Packard Instrument

A new technique for higher counting efficiency in ^3H SPA

Packard now offers a new TopCount Topics application paper (TCA029), which describes the improved performance of this microplate scintillation and luminescence counter for SPA and Cytostar-T assays. Using the newly developed, high-efficiency count mode, the system now increases ^3H counting efficiency by a stated two- to five-fold. This improvement is particularly beneficial for strongly colour-quenched samples in which the maximum effect of the new counting mode is exhibited. Higher counting efficiency may also result in reduced counting times for the popular high-throughput screening applications of SPA.

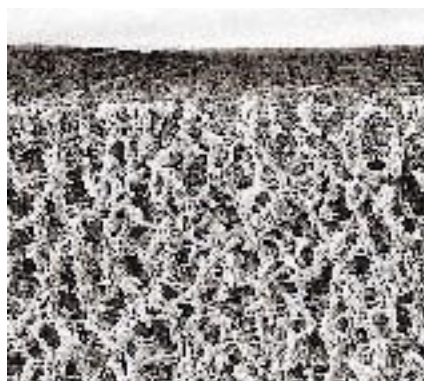
Reader Enquiry No. 114

Pellicon XL 50

From Millipore

New low-binding ultrafiltration membranes

Millipore has released the Pellicon XL 50 tangential flow device with the additional molecular weight cutoffs of the low-binding PL series composite ultrafiltration (UF) membranes. The device is now offered with six composite regenerated cellulose membranes ranging from 5K to 1,000K. They are intended for a variety of process develop-



Going off at a tangent: Millipore's Pellicon XL range covers the firehose from 5 to 1,000K.

ment and sample preparation applications for processing up to 1,000 ml. If high-flux is required, the device can also be supplied with a wide range of Biomax (PES) membranes. For true linear scalability, Pellicon XL devices provide the same flow path, channel length and channel height as the company's established Pellicon 2 cassette products. Primary applications include concentration and desalting of proteins buffer exchange, depyrogenation, clarification and preparation of material for clinical trials.

Reader Enquiry No. 115

Combinatorial chromatography system

From Gilson

An eight-page, four-colour brochure that explains how the system increases throughput, eliminates errors and saves time in the drug discovery process

Special emphasis is given to the system's graphic sample-tracking software, which can save hours of time matching samples, peaks and fractions, says Gilson. On a single screen, it can display a chromatogram, a list of sample descriptions or images of the sample and collected fraction tubes. The user can point and click on a sample-tube image and the corresponding chromatogram and fractions are automatically displayed on the screen. Graphic sample tracking minimizes the need to count events, reducing analysis to a matter of seconds and reducing error. The system offers high capacity, accommodating up to 12 standard or deep-well microplates, for a 1,152 total well capacity. Other options include 13-mm $\times$ 100-mm test tubes, 2-ml vials and 125-ml bottles. Different size racks and collection vessels can be used simultaneously should injection and collection volumes vary. The system features a high level of automation for long, unattended operation. The system utilizes a single instrument for sample injection and fraction collection. As a result, microplates or test tubes remain stationary throughout the process, eliminating the need to move racks or samples manually if reanalysis of purified fractions is required.

Reader Enquiry No. 116

HP 7686 solution-phase synthesizer

From Hewlett-Packard

Automated synthesis and cleanup to accelerate drug discovery

The synthesizer was designed to be a compact tool that can increase the productivity of individual medicinal chemist, allowing them to explore structure-activity relationships in greater depth than has been possible using manual synthesis techniques. The HP 7686 solution-phase synthesizer can produce as many as 200 compounds a week through single-step automated reactions that require minor cleanup routines; or about 60 compounds a week through multistep reaction sequences that include labour-intensive liquid/liquid extraction or solid-phase cleanup. The synthesizer can generate tens of milligrams of these compounds, depending on the chemistry. Some examples of automated organic syntheses that have been performed using the HP synthesizer that include amide bond formations, Suzuki coupling reactions, Grignard and related metal-promoted coupling reactions, enol and enolate rearrangements, aldol and related condensations, Diels-Alder and similar cyclo-addition reactions, Wittig reactions, heterocyclic syntheses, and nucleophilic displacements.

Reader Enquiry No. 117

Hardware

NA mixer

From Biopharma Process Systems

A magnetically coupled NA Mixer from NovAseptic Equipment, available in the UK through Biopharma Process Systems

The mixer is designed for use with all liquid based formulations found in many pharmaceutical and biotech processes, such as injectables, vaccines, infusion solutions,

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READER ENQUIRY NO. 18



Attractive proposition: magnetic mixing is at the core of the new mixer from Biopharma.

plasma fractions, bacteria and cell cultures. The open-structure design of the mixing head permits vigorous mixing or low-volume blending. Most importantly the design also ensures that all surfaces coming into contact with the product, such as the drainable female bearing and drainable internals, are all easily and effectively cleaned *in situ* by spray ball wash. The magnetically powered drive is mounted at the bottom of the chamber and as mechanical seals are not required the problems associated with traditional rotary shaft drives are eliminated. On selected models quick connection of the drive unit makes it easy to disassemble when, for example, autoclaving the vessel, or for using the drive on another vessel. The NA Mixer can handle volumes from 10 to 30,000 l and is available with a choice of mixing heads.

Reader Enquiry No. 118

LipoFast-Pneumatic

From Avestin

For the production of unilamellar liposomes by powered extrusion through a membrane

With this system, lipid emulsion is extruded repeatedly by pneumatic power through a controlled pore diameter membrane. Depending on the formulation, 5–11 passes are usually sufficient to produce a liposome suspension of uniform size, with a stated processing time of less than five minutes. The instrument's stainless steel syringes have a capacity of up to 2.0 ml, making it useful for the preparation of small samples. The LF-P has an extremely low dead volume,



Pore law: liposomes in less than 5 minutes, from the LipoFast-Pneumatic.

allowing for almost complete sample recovery. The LipoFast-Pneumatic has a water bath for product temperature control, which may be used for processing of high transition temperature lipids in a fluid state or to protect temperature sensitive materials. The heat transfer is quick, and the water is easily drained from the instrument after an experiment. The system uses low volume compressed air or gas up to 276 kPa to operate. A pressure higher than 207 kPa is not recommended and may result in membrane rupture.

Reader Enquiry No. 119

Megadac

From Optim Electronics

A CAN-bus interface has been added to this range of data acquisition hardware

The CAN (controller area network) bus technology is rapidly gaining acceptance throughout the automotive, industrial automation and scientific markets. The system uses a BIOLinK interface that plugs directly into the vehicle's standard 55-pin diagnostic plug and can be programmed to select up to eight parameters for recording in parallel with other analog and digital dynamic measurements. The unit is also compatible with a number of variants of CAN-bus, such as ISO-K, V24 and Ford Motor Company's J1850. Although a CAN bus installation typically carries many channels of data that, until now, has been difficult to record and correlate directly with information collected by conventional instrumentation techniques. With BIOLinK, the user is able to input the appropriate parameter identifications (PIDs) using a laptop computer running a simple communications package, such as a Windows terminal. The data corresponding to these PIDs is then converted to a number of analog outputs for recording on a MegaDac data acquisition system. MegaDac evaluation methods include Rainflow, histogram, Rainflow versus mean, Markovian and time-at-level.

Reader Enquiry No. 120

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.



The CAN in a box: the Megadac data acquisition system uses a CAN bus interface.

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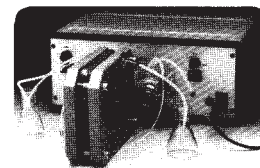
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